

**THE EFFECTS OF ORAL ARGININE ON ITS METABOLIC PATHWAYS IN  
SPRAGUE-DAWLEY RATS**

**DAIN KIM (MSc)**

A Thesis Submitted to the College of Graduate and Postdoctoral Studies in Partial Fulfilment  
of Requirements for the Degree of Master in Anatomy, Physiology and Pharmacology (APP)  
University of Saskatchewan  
Saskatoon

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College of Graduate and Postdoctoral Studies  
University of Saskatchewan  
116 Thorvaldson Building, 110 Science Place  
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## ABSTRACT

Oral arginine supplements are commonly used by the public for their presumed nitric oxide potentiating and vasodilatory role. However, there is a lack of clarity on the physiological impact of oral arginine on its metabolic pathways in the human body. As a versatile molecule, arginine is metabolized by multiple enzymes including arginase, nitric oxide synthase, arginine decarboxylase, and arginine: glycine amidinotransferase. Our lab has recently published a study on the physiological impact of oral arginine at a dose of 500 mg/kg/day administered for 4 weeks in male Sprague-Dawley rats. The present study examined the effects of oral L-arginine and D-arginine in 9-week-old male Sprague-Dawley rats, administered at a higher dose of 1000 mg/kg/day in drinking water for a longer duration of 16 weeks. We measured enzyme expression and activity for different enzymes, and levels of metabolites of the arginine enzymatic pathways in the urine, plasma and various organs of Sprague-Dawley rats. We also measured the expression of the primary arginine transporter, cationic amino acid transporter 1. Oral L-arginine did not alter the expression of cationic amino acid transporter 1 or the levels of arginine and lysine, which use the same transporter, in the plasma and various organs. Oral L-arginine decreased arginase expression in the ileum, and arginase activity in the plasma. It also decreased arginine:glycine amidinotransferase expression in the liver, and creatinine levels in the urine. Similarly, L-arginine supplementation decreased arginine decarboxylase expression in the ileum but increased the expression in the liver with increased plasma total polyamine levels. Interestingly, endothelial nitric oxide synthase expression was significantly increased with oral D-arginine, whereas L-arginine did not cause any significant effects in this pathway, in comparison to control. D-arginine is known to be inactive in the metabolic pathways, but surprisingly, D-arginine supplementation altered the expression of several enzymes and metabolite levels in the treated rats. In conclusion, long term oral supplementation of both L- and D-arginine significantly affected various enzymes and metabolites in the arginine metabolic pathways, as observed with a dose of 500 mg/kg/day for 4 weeks in the previous study from our lab, even though the changes differed in both studies. Determining the physiological impact of oral arginine supplements on the various metabolic pathways of arginine would allow for a better understanding of oral arginine uses, optimum dose and duration, and its safety and efficacy.

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## LIST OF ABBREVIATIONS

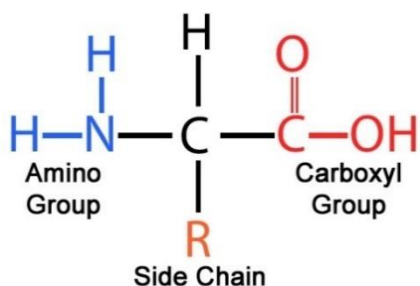
ADC	Arginine decarboxylase
ADMA	Asymmetric dimethyl arginine
ADP	Adenosine diphosphate
AGAT	Arginine:glycine amidinotransferase
AGE	Advanced glycation end products
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAT	Cationic amino acid transporter
cGMP	Cyclic guanosine monophosphate
D-Arg	D-arginine
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
GAMT	Guanidinoacetate <i>N</i> -methyltransferase
GC	Guanylate cyclase
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GTP	Guanosine triphosphate
h	Hour
HR	Heart rate
iNOS	Inducible nitric oxide synthase
L-Arg	L-arginine
MAP	Mean arterial pressure
MELAS	Mitochondrial encephalomyopathy lactic acidosis, and stroke-like episodes
MG	Methylglyoxal
min	minutes
nNOS	Neuronal nitric oxide synthase

NO	Nitric oxide
NOHA	<i>N</i> <sup>ω</sup> -hydroxy-L-arginine
NOS	Nitric oxide synthase
OAT	Ornithine aminotransferase
ODC	Ornithine decarboxylase
PDE <sub>i</sub>	Phosphodiesterase inhibitors
PVDF	Polyvinylidene difluoride
SD	Sprague Dawley
SDS	Sodium dodecylsulfate
sec	Seconds
SEM	Standard error of mean
T2DM	Type 2 diabetes mellitus
TBST	Tris buffered saline with tween

## CHAPTER 1: BACKGROUND

### 1.1 Amino Acids

Amino acids are organic monomers of proteins that consist of an amino group, a carboxylic group, and a side chain group, as shown in Figure 1. With the exception of glycine, all amino acids carry a chiral carbon and come in two mirror images called enantiomers, commonly referred to as optical isomers. The two forms are designated as L- and D-isomer, where the L- and D-configuration of amino acid is determined by the location of the amino group linked to the chiral carbon in the Fischer projection formula. L-amino acid has an amino group on the left side of the chiral center, and D-amino acid has it on the right side (Grishin et al., 2020). The majority of the amino acids in the body that serve as building blocks for protein only exist in the L-isoform, making the D-isomer appear as physiologically inert. Extending from this fact, it has been noted that the enzymes for protein synthesis have likely adapted to only using the L-configuration of amino acids (Reddy, 2020). The exact reasoning behind the predominance of this enantiomer has not been clearly established yet, although there are various studies and theories that have come forward that are mainly associated with evolutionary selectivity (Grishin et al., 2020).



**Figure 1. Structure of an amino acid**

(Image Source: [http://www.astrochem.org/sci/Amino\\_Acids.php](http://www.astrochem.org/sci/Amino_Acids.php))

Though there are D-amino acid containing peptides that exist in eukaryotes, these peptides are not directly produced from the ribosomes with the traditional protein synthesis machinery, but rather using different mechanisms such as post-translational modification (Grishin et al., 2020). By the process of racemization with racemase and D-amino acid oxidase, D-amino acids can be converted into L-amino acids, which makes the presence of D-amino acid in food an indicator for certain occurrences, like fermentation or contamination (Genchi, 2017). It has been revealed that some D-amino acids in fact carry out important physiological functions in the endocrine system

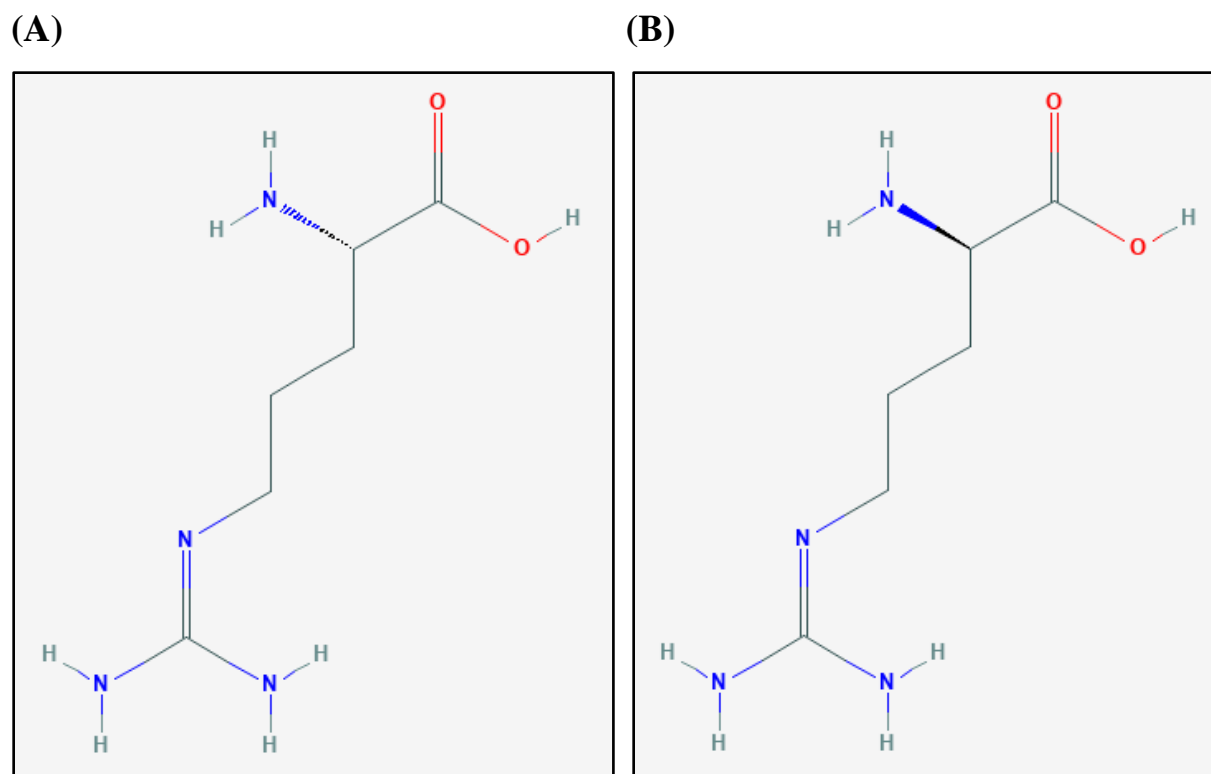
and the nervous system of mammals, although there is still a huge gap of knowledge on the effects of D-amino acids, in comparison to L-amino acids (Kiriya and Nochi, 2016).

There are 20 amino acids that are known to build proteins in the body and of these, 9 of them are classified as essential amino acids. This is referred to as any amino acid that is unable to be synthesized endogenously to fulfill the optimal physiological demand, and thereby needs to be supplied from the diet (Lopez and Mohiuddin, 2021). On the other hand, amino acids that are referred to as being non-essential are sufficiently produced in the body. Conditionally essential amino acids, also known as semi-essential amino acids, are normally synthesized at a sufficient level in healthy adults, but the synthesis is compromised in specific populations under certain conditions, such as during infancy and for people in times of physiological stress or critical illness (Morris et al., 2017). Arginine, which is the core focus of this research study, is one of the conditionally essential amino acids that is synthesized insufficiently in people under certain conditions including renal or intestinal dysfunction (Morris Jr., 2007). Arginine plays a variety of crucial roles in the body, which will be discussed and examined in depth as a part of this research project.

## 1.2 Arginine Overview

Arginine (Fig. 2) is a versatile, semi-essential amino acid that contributes to the synthesis of many biologically important molecules, by being a substrate in various enzymatic pathways (Böger, 2007). Some products of arginine metabolism include nitric oxide (NO), urea, and creatine (Wu and Morris Jr, 1998). As mentioned earlier about amino acids, from the L- and D-isomers of arginine (Fig. 2), D-arginine (D-Arg) is known as the metabolically inactive isomer (National Center for Biotechnology Information, <https://pubchem.ncbi.nlm.nih.gov/compound/D-Arginine>), thereby the physiological effects of arginine are primarily associated with just L-arginine (L-Arg). Free arginine in the body come from several sources including endogenous synthesis, the diet, and intracellular protein turnover (Morris Jr., 2007). In healthy adults, the normal plasma arginine levels are maintained in the range of 80 – 120  $\mu\text{mol/L}$  (Morris Jr., 2007). As with any person with arginine deficiency, arginine supplementation is necessary for growing adolescents (National Center for Biotechnology Information, <https://pubchem.ncbi.nlm.nih.gov/compound/Arginine>), and physiologically compromised individuals. It has been reported that preterm infants tend to have arginine deficiency (Becker et al., 2000), which is characterized by hyperammonemia and

multi-organ dysfunction (Wu et al., 2004). A study involving individuals with genetic mutation that leads to defective dietary uptake and reabsorption of L-Arg demonstrated that these patients had significantly lower vasodilatory effects and platelet counts in the blood in comparison to the controls, and showed myocardial ischemia with exercise testing (Loscalzo, 2001). Together, these physiological differences depict the importance of L-Arg nutrition in the body, especially in association with the role of NO, which is a metabolite of one of the major arginine pathways, that will be thoroughly discussed later.



**Figure 2. Structures of L-arginine and D-arginine**

(A) L-arginine and (B) D-arginine.

Source: [L-Arginine | C6H14N4O2 - PubChem \(nih.gov\)](#); [D-Arginine | C6H14N4O2 - PubChem \(nih.gov\)](#)

Fortunately, with a wide variety of dietary sources of arginine, including nuts, milk products, and legumes, fulfilling the metabolic demands of the body is made easy by simply adjusting the diet (McNeal et al., 2016). A typical western diet provides approximately 25 to 30% of total arginine levels in the blood (Rosenthal et al., 2016). In support of this statement, a research study



done on healthy male subjects in their early twenties, suggested that homeostasis in arginine levels is largely established by the diet (Castillo et al., 1993a).

### 1.3 Pharmacokinetics of Arginine

#### 1.3.1 Absorption and Bioavailability

Arginine that comes from the diet gets absorbed in the splanchnic area of the body, through enterocytes (Castillo et al., 1993b) at the jejunum and ileum (Pahlavani et al., 2014). Specifically in the epithelial lining of the small intestine, there are brush borders that consist of amino acid transporters for dietary absorption (Souba and Pacitti, 1992). The transport system primarily responsible for selective uptake of cationic amino acids like arginine in the small intestine is referred to as the  $y^+$  system, which comprises of the cationic amino acid transporter (CAT) family (Devés and Boyd, 1998). Within this family, there are several CAT isoforms, and it has been suggested that each of the CAT isoforms has affinity to one of the three isoforms of nitric oxide synthase (NOS) enzymes (Schwartz et al., 2006). CAT-1 is known to be specifically responsible for the transport of arginine to endothelial nitric oxide synthase (eNOS) for its use (Schwartz et al., 2006). With saturation of the available transporters, such as with increase in post-prandial arginine levels, passive diffusion may occur for the uptake of arginine (Tangphao et al., 1999a). An issue with the amino acid transporters is that basic, cationic amino acids like arginine and lysine, use and compete for the same type of transporters, affecting the uptake of each other (Wu et al., 2009). In relation, it has been suggested that excess arginine intake leads to disrupted homeostasis of amino acids, causing adverse effects such as impairment in growth (Edmonds et al., 1987). The remaining free arginine in the body is filtered by the kidney and almost entirely gets reabsorbed through its transporters in the proximal tubule (Brosnan and Brosnan, 2004).

Although arginine needs are readily met through the diet, approximately 40% of it undergoes first pass metabolism in the gut, limiting the bioavailability of free arginine in the body to enter the systemic circulation (Wu et al., 2009). A pharmacokinetic study of arginine supplementation in healthy adults (Tangphao et al., 1999b) found that following administration of 10 g of oral arginine in solution form, the absolute bioavailability in healthy volunteers turned out to be only around 20%, but with a broad range from 5 to 50%. This result indicating low availability is not surprising since free arginine interacts with multiple enzymes and goes through extensive metabolism before reaching the circulation. However, several other studies reported higher

bioavailability of oral arginine, such as in an arginine study involving hypercholesterolemic population where they reported the bioavailability to be around 40 to 50% (Tangphao et al., 1999a). One arginine pharmacokinetic study reported even higher bioavailability of oral arginine in healthy population following single oral administration, where the bioavailability was around 70%, ranging from 51 to 87% (Bode-Böger et al., 1998). As the variance between the studies suggest, the exact pharmacokinetics regarding the bioavailability of arginine is not well-established yet, but it is crucial to appreciate how important bioavailability is in understanding the role of oral arginine supplements in the body. The physiological effects of arginine such as vasodilation is known to be highly associated with its concentration in the plasma (Bode-Böger et al., 1998). Thereby, bioavailability is an important factor when considering the therapeutic effects of oral arginine supplements in the body.

### 1.3.2 Distribution

The bioavailability of arginine is directly linked with its distribution, as greater bioavailability in the blood allows for greater tissue and organ distribution. Studies that have been published on arginine pharmacokinetics have reported varying results in its distribution in the body. A study involving autoradiography with biochemical analysis recorded the radioactivity in organs of mice at various time intervals following intravenous injection of radio-labelled arginine (Goto, 1989). This study reported low distribution of arginine in the brain, lungs, and testes, and highest uptake by the pancreas at all time intervals.

In contrast, there have been studies reporting otherwise, for instance, that both oral and intravenous arginine administration significantly increased the level of free arginine in the liver, testes, and the brain (Campistron et al., 1982). One important result that was noted from this study is that, for testes and the brain, the oral route of administration in comparison to intravenous route led to higher distribution of arginine. In another study involving patients with multiple myeloma, it was demonstrated that there was no preferential distribution of guanido-labeled arginine in any particular tissues from the nineteen types that were examined (Frondoza et al., 1980). In the latter study, plasma protein binding of arginine was also reported, which is a factor that affects distribution. The extent of binding varied among the patients, where interestingly, the individuals with advanced disease status showed high plasma protein binding of arginine with the majority

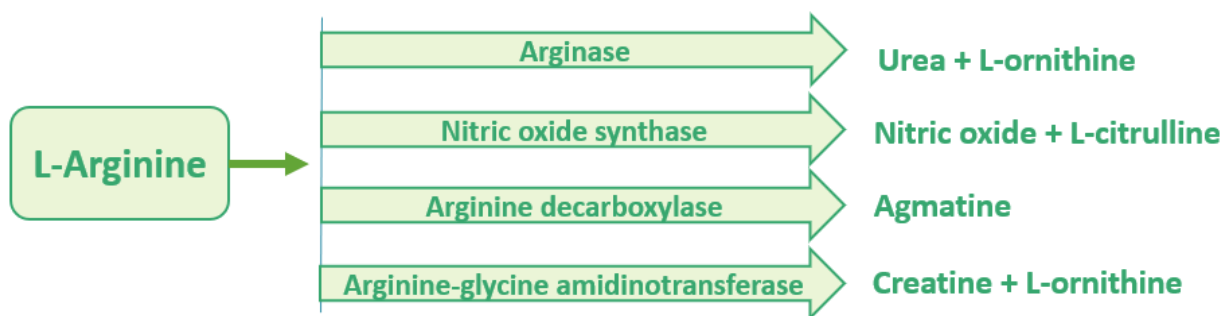
lasting for 7 to 10 days, and the rest showed 42% as maximum binding, with fast rate of disappearance of the tracer of bound arginine.

With limited and varying results of research on arginine distribution in the human body, more studies need to be carried out to establish a clear understanding on the pattern of distribution following oral arginine administration. Filling this gap of knowledge would be helpful in understanding how much the arginine dose reaches the target site, in association to the administration route, safety and efficacy, and to see the overall picture of arginine pharmacokinetics from supplementation.

### 1.3.3 Metabolism: Synthesis and Enzymatic Pathways

There are several mechanisms for arginine synthesis in the body. Some amino acids such as proline and glutamine are interconvertible with arginine through a series of enzymatic steps, where both amino acids convert to a common intermediate, pyrroline-5-carboxylate, to ultimately convert to arginine (Wu et al., 1997). This intermediate gets further converted into ornithine by the key enzyme, ornithine aminotransferase, and then to citrulline, before reaching the final step where arginine is formed from arginosuccinate with the action of arginosuccinate lyase (Wu et al., 1997). In addition to proline and glutamine metabolism, arginine is made from a variety of other sources that involve citrulline production, including the NOS pathway and the enzymatic breakdown of asymmetric dimethyl arginine (ADMA) (Morris Jr, 2004). In the urea cycle however, arginine is synthesized from citrulline as an intermediate, but does not contribute to the overall levels of free arginine in the body as it gets readily converted into ornithine and urea as a byproduct (Watford, 2003). Out of all the sources for the synthesis of free arginine in the body, the intestinal-renal axis remains the key site in producing free arginine in the body (Wu and Morris Jr, 1998). In brief, the metabolism of mainly glutamine in the epithelial cells of small intestines forms citrulline as explained above and releases it in the circulation where it travels in the blood and eventually gets taken up by the proximal tubules of the kidney (Morris Jr, 2004). In the kidney, conversion of citrulline to arginine occurs, and the newly synthesized arginine gets released back into the bloodstream (Morris Jr, 2004). This is the reason why arginine supplements become necessary in individuals who have renal or intestinal dysfunction. It has been found that approximately 85% of citrulline synthesized from the intestines is taken up by the proximal tubules of kidney to produce arginine (Brosnan and Brosnan, 2004).

As a versatile molecule, arginine gets extensively metabolized through multiple metabolic pathways in mammalian cells. A study indicated that after around 30 min of arginine administration by injection, greater than 90% of labelled arginine was metabolized from the intact form (Frondoza et al., 1980). As illustrated in Figure 3, arginine acts as a metabolic substrate for arginase, NOS, arginine:glycine amidinotransferase (AGAT/GATM), and arginine decarboxylase (ADC) (Morris Jr, 2004). By these enzymes, arginine leads to the production of many biologically important molecules in the body, including urea, NO, agmatine and creatine.

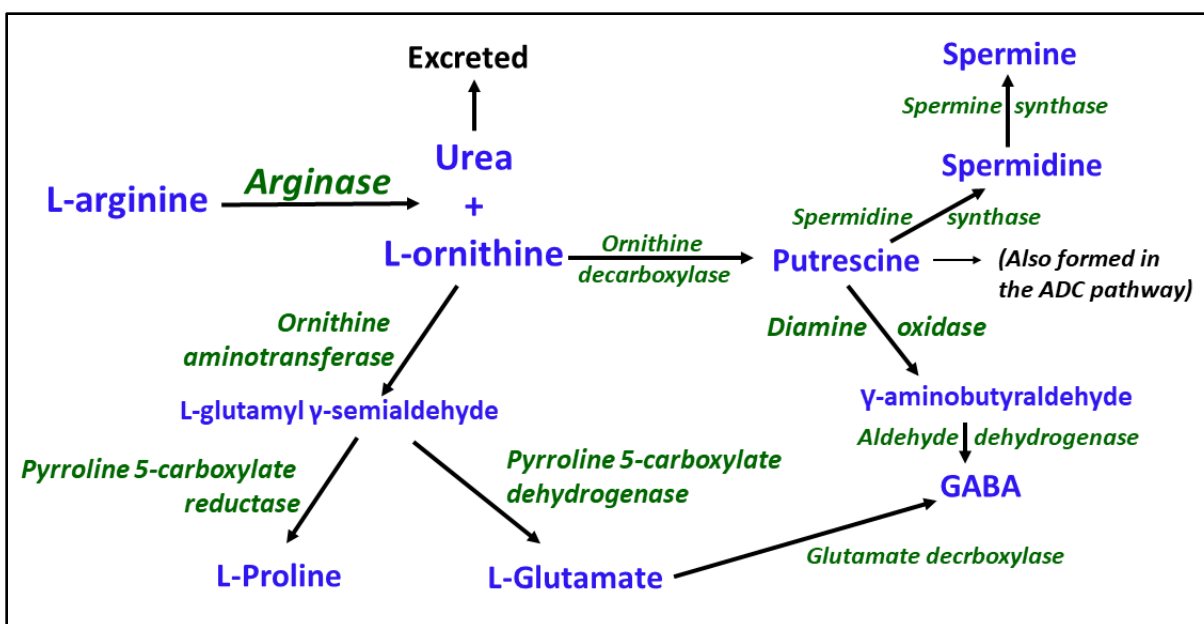


**Figure 3. Main enzymatic pathways and metabolic products of L-arginine**

#### 1.3.4 Arginase Pathway

The arginase pathway leads to formation of urea plus L-ornithine from L-arginine (Fig. 4). Ornithine is a nonprotein amino acid that leads to the formation of proline and polyamines (Ash, 2004). Thus, arginase is an important enzyme of the urea cycle. As commonly known, the role of urea cycle is to detoxify ammonia and convert it into inert water-soluble urea for excretion.

In mammals, the arginase enzyme exists in two isoforms, arginase type I and type II. The two isoenzymes are encoded separately by different genes and though they share comparable enzymatic properties, factors such as tissue distribution and expression regulation are different (Wu and Morris Jr, 1998). Compartmentalization of arginase in mammalian cells may affect arginine metabolism through enzymes that act upon ornithine. Arginase I is usually colocalized with ornithine decarboxylase (ODC) in the cytosol, whereas arginase II is colocalized with ornithine aminotransferase (OAT) in the mitochondria (Li et al., 2001). ODC converts ornithine into polyamines, whereas OAT converts ornithine into proline (Fig. 4). With this information, as illustrated in Figure 4, arginase I may be considered to preferentially lead to polyamine synthesis and arginase II may preferentially lead to glutamate and proline production.



**Figure 4. Arginase metabolic pathway of L-arginine.** Arginase converts L-arginine to urea plus L-ornithine. Urea is inert and water soluble and easily excreted by the kidneys. L-ornithine can be converted to L-proline, L-glutamate or putrescine depending on further enzymatic pathway. Putrescine forms polyamines such as spermidine and spermine or GABA ( $\gamma$ -amino butyric acid), which can also come from L-glutamate. (Figure adapted from Morris Jr. SM., 2007)

Arginase I is also referred to as the liver arginase, as this mainly exists in the cytosol of hepatic tissues acting as the final enzyme of the urea cycle, though it is also expressed in some extrahepatic tissues in low levels (Wu and Morris Jr, 1998). Arginase I in the liver is responsible for the detoxification and excretion of waste nitrogen, specifically ammonia, in the form of urea, and the activity of this isoenzyme is known to make up for the majority of the total arginase activity in the body (Durante et al., 2007). As well, some studies documented that this isoenzyme activity can be upregulated by some hemodynamic and growth factors in the body, which induce this enzyme expression in vascular cells (Durante et al., 2007).

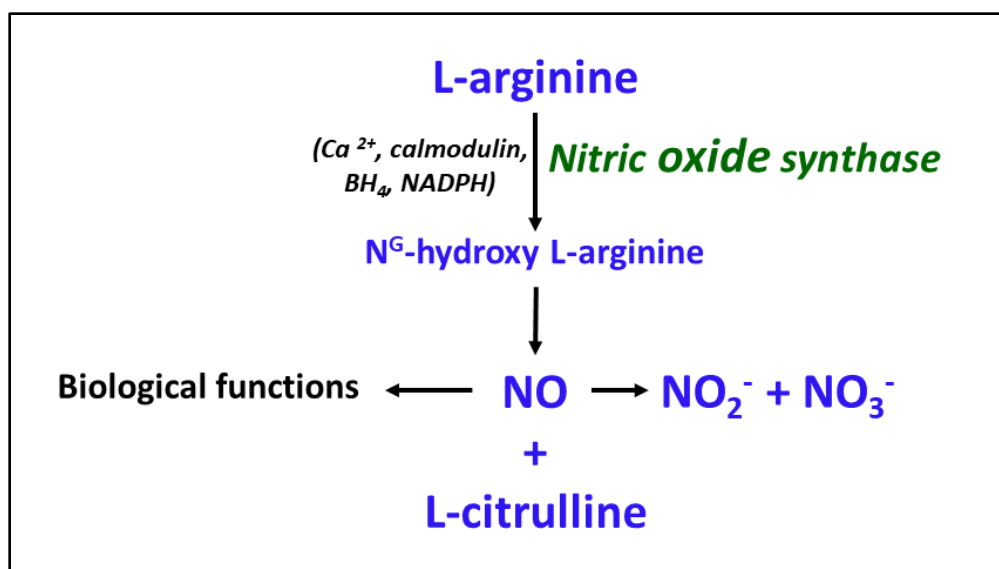
In contrast, arginase II is widely distributed in the mitochondria of numerous parts of the body such as the small intestine, brain, kidney, macrophages, prostate, mammary glands and some vasculature, but at lower levels (Wu and Morris Jr, 1998; Durante et al., 2007). A study found that in comparison to other organ tissues, the expression of arginase II was high in the kidney and the pancreas, moderate in the intestines, and very low in the liver (Choi et al., 2012). The exact

function of arginase II in non-hepatic tissues has not been clearly established yet, but the activity contributes to the formation of polyamines and other amino acids, such as proline and glutamate (Wu and Morris Jr, 1998; Ash, 2004). As mentioned earlier, ornithine gets converted into polyamines, by first getting metabolized into putrescine by ODC, which further yields other polyamines (Stechmiller et al., 2005). Proline is responsible for biosynthesis of milk protein in mammary glands during lactation, and polyamines play a role in cell proliferation and growth (Ash, 2004). In times of infection or inflammation, arginase expression and activity are elevated, causing more ornithine production and subsequently more synthesis of proline and polyamines. This increase in synthesis is able to facilitate proliferation of cells and collagen production which take crucial part in various physiological processes and disorders, such as wound healing, inflammation, infection and fibrotic disorders (Morris Jr, 2004).

Out of the four main enzymatic pathways of arginine, the arginase pathway is known to be the most important one for the catabolism of arginine in mammals (Wu and Morris Jr, 1998). Some studies suggest that the metabolism of arginine through arginase I in the liver, rather than endogenous synthesis, is the regulating factor for total arginine levels and homeostasis in the body (Castillo et al., 1994). With arginine administration, it was found that the rate of urea synthesis was significantly higher than the rates of synthesis for the metabolites of other arginine pathways such as NO and creatine (Wu and Morris Jr, 1998). As well, a labeled arginine study determined that radioactivity from tagged arginine in rat liver was significantly lower than that of other organ tissues, with a very fast rate of arginine being replaced in this tissue in particular, which accompanies the idea that the bioavailable arginine readily gets taken up and used up by arginase as a part of the urea cycle in the liver (Frondoza et al., 1980).

### 1.3.5 Endothelial Nitric Oxide Synthase Pathway

Out of the four arginine pathway enzymes, NOS has been most extensively studied for its regulation and role in the arginine metabolic pathways (Wu and Morris Jr, 1998). As shown in Figure 5, NOS facilitates the conversion of L-Arg into NO and L-citrulline. L-citrulline is an intermediate of the urea cycle and as mentioned previously, it is able to be converted into L-Arg through a series of enzymatic steps. Interestingly, it is used widely as a supplement as well, as it is known to act as a better and more effective precursor for NO functions in the body (Kaore et al., 2013).



**Figure 5. Nitric oxide synthase enzymatic pathway of L-arginine metabolism.** NOS enzymes, of which there are three isoforms, convert L-arginine into NO and L-citrulline, with the help of calcium ( $Ca^{2+}$ )-calmodulin, tetrahydrobiopterin ( $BH_4$ ), and nicotinamide adenine dinucleotide phosphate (NADPH). NO has various biological functions and is metabolized into nitrite ( $NO_2^-$ ) and nitrate ( $NO_3^-$ ). L-citrulline is recycled back into L-arginine and has other metabolic fates. (Figure adapted from Morris Jr. SM., 2007)

NO is a potent vasodilator that mediates a variety of important functions in the body. It is produced by the vascular endothelium, certain neurons and macrophages in response to a variety of agonists/stimuli including shear stress and bacterial lipopolysaccharide (LPS) (Endemann and Schiffrin, 2004). Some of the physiological roles that it plays include vasodilation, platelet antiaggregatory, neuronal long-term potentiation, non-adrenergic non-cholinergic (NANC) neurotransmitter, immune responses and inflammation (Wu and Morris Jr, 1998; Endemann and Schiffrin, 2004). The vasodilatory effect of NO is the primary reason for which arginine supplements are being taken by a wide range of population nowadays. Several studies have shown that NO mediates neurotransmission in learning and memory, and that it also plays a neuroprotective role in the central nervous system (Böhme et al., 1993; Paakkari and Lindsberg, 1995). The metabolic end products of the eNOS pathway, nitrates and nitrites, are recognized and being used as effective markers for the level of NO produced, rather than using NO itself, because

NO has a very short half-life (5-6 sec), and the metabolites have been found to be stable as its indicators (Lundberg, 2006; Giustarini et al., 2008).

The NOSs exist in three isoforms dispersed throughout the body that differ by various factors including genetic makeup, localization and regulation (Alderton et al., 2001). The names of each of the isoforms have been created according to the site that they were first discovered in the body (Böger, 2007).

The first isoform, neuronal NOS (nNOS), also referred to as NOS-I, was first found and is predominantly expressed in neuronal tissues of both the central and peripheral nervous system (Alderton et al., 2001). It is believed to be mainly expressed in the neurons of the brain and the enteric nervous system in a constitutive manner, but it also has been found in many other locations, such as adrenal glands, organ epithelial cells, kidney, vascular smooth muscle, kidney and the pancreas, and the skeletal muscle (Förstermann and Sessa, 2011). The physiological functions of nNOS involve regulating synaptic transmission and plasticity in the long term which is associated with memory formation, and it may contribute to regulating vascular tone and systemic blood pressure as well, though the extent of effect is unclear in comparison to eNOS, which is conventionally believed to be the principal enzyme for vasodilation (Förstermann and Sessa, 2011). NO produced by enteric neurons has been found to relax the gastrointestinal smooth muscle (Bult et al., 1990; Desai et al., 1991).

Another isoform is the inducible NOS (iNOS), also known as NOS-II, which plays an important role in immune defense. As the name states, iNOS expression is inducible by various agents such as bacterial LPS and cytokines, rather than it being expressed in particular cells in the body. Although iNOS is capable of being induced in any region of the body, the main cell type is macrophages (Förstermann and Sessa, 2011). iNOS activation leads to inflammatory reactions and production of large amounts of NO to exert cytotoxic effects on bacteria, parasites, and cancer cells (Nathan and Hibbs, 1991). Something to consider with the effects of iNOS is that it may also damage healthy cells and tissues if it produces lots of NO in the wrong tissue, for example in septic shock, where it causes unmanageable vasodilation and hypotensive shock which can prove fatal (Petros et al., 1991).

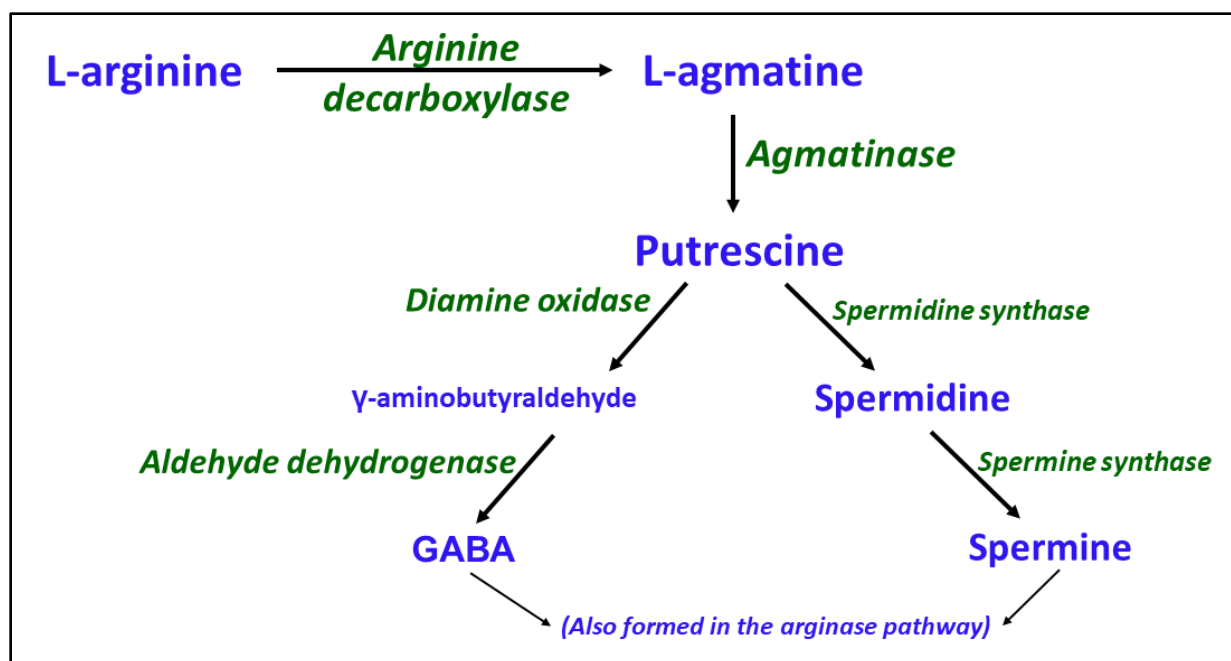
Lastly, endothelial NOS (eNOS), also called NOS-III, is mostly expressed in endothelial cells, and exhibits numerous cardiovascular roles. Out of the three isoforms of NOS, eNOS is the focus of this research study. eNOS produces NO in a pulsatile manner in order to regulate blood flow by



vasodilation, protect from blood clotting by exerting anti-platelet effects and inhibit vascular inflammation (Förstermann and Sessa, 2011). The function of NO is the primary reason why arginine supplements are popular nowadays. NO production *via* eNOS in the endothelium causes endothelium-dependent vasodilation, which occurs in response to signaling molecules such as bradykinin and acetylcholine, or mechanical forces (Shesely et al., 1996). An increase in calcium levels in the endothelial cell in response to the factors mentioned above, causes binding of eNOS to calmodulin, which leads to the activation of eNOS to produce NO (Förstermann and Sessa, 2011). NO then directly diffuses into the vascular smooth muscle to stimulate guanylate cyclase (GC), converting guanosine-triphosphate (GTP) into cyclic guanosine-monophosphate (cGMP) to cause vasodilation (Archer et al., 1994). Due to the protective and regulatory role that NO has in the body, especially in the vasculature through eNOS activity, NO deficiency is the main reason for endothelial dysfunction which is believed to be a precursor of many cardiovascular diseases, such as hypertension and atherosclerosis (Förstermann and Münzel, 2006).

#### 1.3.6 Arginine Decarboxylase Pathway

In addition to the arginase pathway, the ADC pathway (Fig. 6) is another metabolic pathway of arginine that leads to the production of polyamines, by first converting L-Arg into agmatine with ADC (Fig. 6), followed by hydrolysis of agmatine into putrescine by agmatinase (Wang et al., 2014a). Both, the ADC and agmatinase enzymes are highly expressed in the brain and are together responsible for facilitating the production of polyamines as a part of the pathway referred to as “agmatine pathway” (Halaris and Plietz, 2007). Another enzyme, ODC serves a function that aligns with ADC in generating polyamines in the body as illustrated in Figure 4, by converting ornithine into putrescine (Halaris and Plietz, 2007).



**Figure 6. The arginine decarboxylase (ADC) metabolic pathway of L-arginine.** ADC forms L-agmatine from L-arginine, which is further metabolized to putrescine by agmatinase. Just like in the arginase pathway putrescine can be converted into the polyamines, spermidine and spermine, and into GABA ( $\gamma$ -amino butyric acid). (Figure adapted from Morris Jr. SM., 2007)

Putrescine is a polyamine that acts as a precursor to other polyamines such as spermidine and spermine (Gao et al., 2009). ADC and its branch of arginine metabolic pathway had been in controversy in terms of its existence and physiological effects in mammals, as for a long time it was generally believed that ADC only exists in bacteria and plants (Coleman et al., 2004). After numerous studies and findings on ADC using a variety of research techniques, ADC has been scientifically accepted as a mammalian enzyme producing agmatine (Coleman et al., 2004).

Polyamines are important regulators of mammalian cell growth, conceptus development, and protein synthesis (Mandal et al., 2013). There are numerous research studies that focused on the effect of polyamines on mammalian cell cycle, which demonstrated that polyamines are highly correlated with cell proliferation and tissue growth (Pegg and McCann, 1982).

The direct metabolite of ADC, agmatine, is an amine mainly located in the brain regions, acting as an intermediate for polyamine synthesis (Halaris and Plietz, 2007). The exact physiological roles of agmatine, aside from polyamine synthesis, are not clearly understood yet, but there have been many suggestions with respect to its function in the body. It has been suggested that agmatine

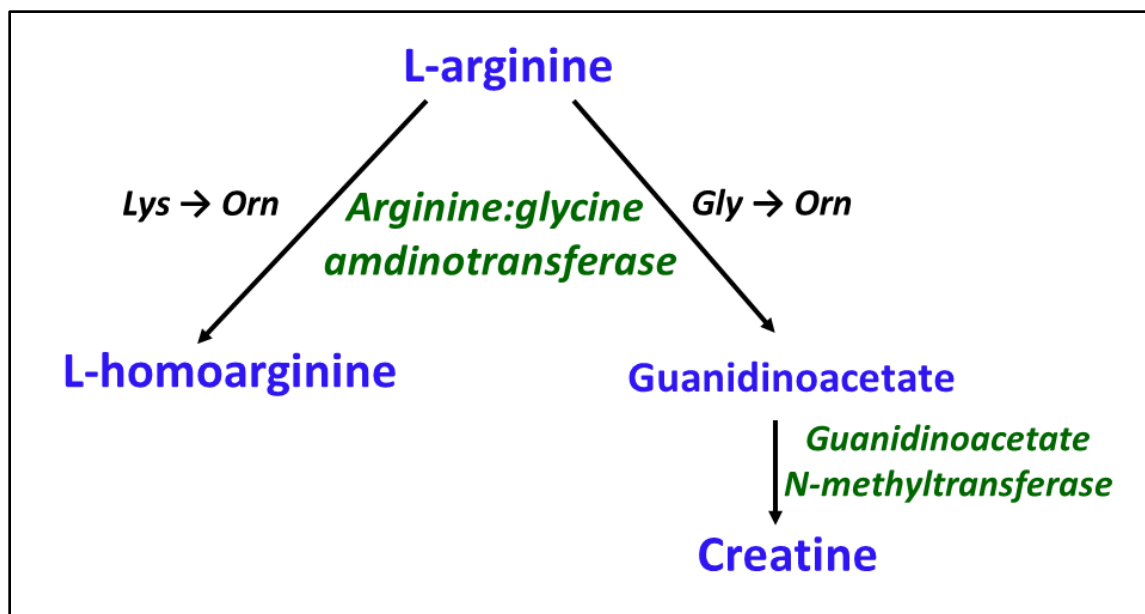
may potentially serve important roles in cell proliferation and inflammation, and neuromodulation (Reis and Regunathan, 1998; Satriano et al., 1998; Zhu et al., 2004). Interestingly, agmatine may be counterintuitive by causing a decrease in cellular polyamine levels by activating antizymes and inhibiting ODC activity which normally contributes to polyamine biosynthesis (Satriano et al., 1998).

One of the primary reasons for the notion that agmatine may have neuromodulating functions was due to the discovery that agmatine was made and stored in neurons, and released from synaptic vesicles (Feng et al., 1997; Goracke-Postle et al., 2006). Several studies have further demonstrated that agmatine acts as a cell-signaling ligand for various receptors including alpha-2 adrenergic, imidazoline, and glutamatergic NMDA receptors (Li et al., 1994; Piletz et al., 1995), and that it modulates the release of various hormones and transmitters (Li et al., 1994; Kalra et al., 1995). Due to these reasons, a name for its potential actions on cellular and neural transmission was established as “agmatinergetic transmission system”, to describe the actions of agmatine as a neurotransmitter (Reis et al., 1998). Furthermore, it was reported that at high levels, agmatine irreversibly blocks nNOS activity by half of its normal activity (Galea et al., 1996; Demady et al., 2001) and downregulates iNOS activity as well (Satriano et al., 2001). As mentioned earlier, the exact physiological mechanism and effects for these reported agmatine functions remain controversial and unclear to this day, but what we do know is that agmatine is capable of acting on a variety of receptors in the body, thereby serving certain physiological roles that need to be further studied upon.

### 1.3.7 Arginine:Glycine Amidinotransferase Pathway

Arginine:glycine amidinotransferase (AGAT/GATM) is the rate-limiting enzyme for creatine synthesis in the body (Fig. 7), as a part of the arginine pathways (Wu and Morris Jr, 1998). AGAT expression and distribution is limited to mainly the kidney and pancreas, and to a lower degree, in the liver (Walker, 1979; McGuire et al., 1984). Creatine biosynthesis *via* the AGAT pathway occurs in two steps (Brosnan and Brosnan, 2004). First, AGAT catalyzes the transfer of a guanidino group from arginine to glycine, producing guanidinoacetate and ornithine as a result (Fig. 7). Secondly, guanidinoacetate N-methyltransferase (GAMT) catalyzes methylation of guanidinoacetate using S-adenosylmethionine, producing creatine and S-adenosylhomocysteine (Fig. 7) (Brosnan and Brosnan, 2004). GAMT is predominantly expressed mainly in the liver and

pancreas (Braissant et al., 2005). Considering the limited enzyme expression, guanidinoacetate is mainly produced in the kidney by AGAT, and moves to the liver to be methylated to become creatine by GAMT. Creatine levels in the liver have been observed to be very low, which suggests that after synthesis, it gets distributed to other tissues (Wyss and Kaddurah-Daouk, 2000).



**Figure 7. Arginine:glycine amidinotransferase enzymatic pathway for L-arginine metabolism.** Arginine:glycine amidinotransferase (AGAT/GATM) catalyzes the conversion of L-arginine into guanidinoacetate and then into creatine. It can also form L-homoarginine.. (Figure adapted from Morris Jr. SM., 2007).

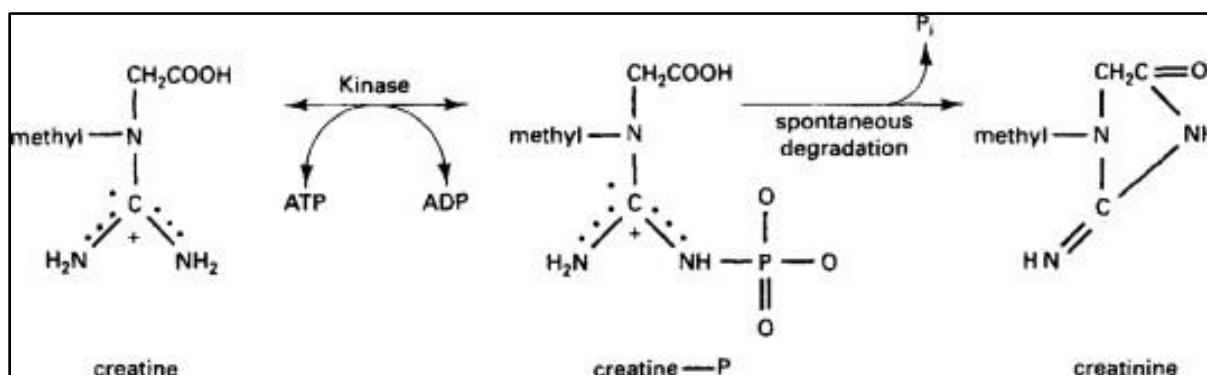
The newly synthesized creatine from the liver enters the circulation and is readily distributed into skeletal muscle and nerves (Wu and Morris Jr, 1998). Specifically, it gets actively taken up from the blood predominantly by muscular tissues against a concentration gradient, *via* the Na<sup>+</sup>- and Cl<sup>-</sup>-dependent creatine transporters that are located along the plasma membranes (Wyss and Kaddurah-Daouk, 2000).

Creatine is a very important molecule that regulates the levels of adenosine triphosphate (ATP) in the body, and with phosphocreatine and creatine kinase, it buffers energy needs in various tissues and is known to boost energy levels during rapid, high intensity exercise (Michel, 2013; da Silva et al., 2014). Creatine couples with a phosphoryl group released from ATP to become phosphocreatine with the catalytic help of creatine kinase, and about two thirds of creatine content

in muscles exists in the form of phosphocreatine (Kreider et al., 2017). As presented in Figure 8, creatine kinase works bidirectionally, thereby when ATP is needed, it facilitates hydrolysis of phosphocreatine into creatine and phosphoryl group to release energy that is used to form ATP with adenosine diphosphate (ADP) and the phosphoryl group (Kreider et al., 2017). The function of creatine differs for fast- and slow-twitch skeletal muscles. For fast twitch work that requires short burst of energy, stored phosphocreatine gets used with high level of activity of creatine kinase to readily regenerate depleted ATP, and the levels of ADP and ATP, as well as the phosphorylation state of creatine are kept at near equilibrium. On the other hand for slow twitch work of skeletal muscles, the site using ATP requires more of a continuous transport of high energy phosphates (Wyss and Kaddurah-Daouk, 2000).

In addition to the role of creatine in forming phosphocreatine, it is also known to link cellular sources of ATP synthesis such as glycolysis and oxidative phosphorylation of mitochondria, with sites that are in need of ATP energy, such as ATPases (Wallimann et al., 2011; Schlattner et al., 2016; Ydfors et al., 2016), which adds on to creatine function of buffering energy needs.

Creatine synthesis in the body is crucial since both creatine and creatine-phosphate spontaneously get metabolized into creatinine, as shown in Figure 8, at a constant rate of around 2% per day, which gets excreted by the kidneys in the urine, requiring replacement for the consistent loss (Wyss and Kaddurah-Daouk, 2000; da Silva et al., 2009). Creatinine is widely used as a clinical indicator for kidney health and function, as it is excreted unchanged by the kidney and allows for easy detection of the glomerular filtration rate (Levey et al., 1988).



**Figure 8. Creatine metabolism**

(Image Source: <https://www.sciencedirect.com/topics/nursing-and-health-professions/creatine-phosphate>)

### 1.3.8 Interaction between Nitric Oxide Synthase and Arginase

As L-Arg serves as a common substrate for both eNOS and arginase in the body, these enzymes compete and interfere with the activity of one another (Wu et al., 2009). Interestingly, L-Arg has higher affinity to NOS in reference to a much lower  $K_m$  value for NOS than arginase, but the  $V_{max}$  of arginase is a lot higher than NOS, indicating faster maximum catalytic rate of reaction with L-Arg, affecting arginine bioavailability for NOS (Wu and Morris Jr, 1998). The difference in these enzymatic factors of the two suggests that L-Arg is used up by both enzymes at a relatively similar rate. Considering this and the potential impact of competing for L-Arg, the enzymatic interplay between NOS and arginase has been a focus of research. There are numerous studies which demonstrated that inhibiting arginase increases production of NO (Chang et al., 1998; Bruch-Gerharz et al., 2003; Cuihua et al., 2004), and overexpressing arginase decreases production of NO (Li et al., 2001; Meurs et al., 2002; Cuihua et al., 2004).

The mechanism of interaction between NOS and arginase goes beyond the competitive nature for a common substrate. As a part of the NOS pathway shown in Figure 4, a metabolite called  $N^G$ -hydroxy-L-arginine (NOHA) strongly inhibits arginase enzymes (Boucher et al., 1994), increasing arginine availability for NO production in various locations such as the liver and macrophages. Furthermore, NO production can also be interrupted by arginase activity of repressing mRNA translation of iNOS, and this inhibition has been reported to be fairly selective, in that arginase inhibited only half of protein synthesis overall, while iNOS protein expression was completely inhibited (Lee et al., 2003).

Some questions that remain are how and at what dose of regular consumption of oral supplements of arginine would physiologically affect the two enzymes, for instance, causing downregulation in one of the two. This is important, because many people in present days take oral arginine supplements for the presumed NO function in the body, thus, if at a certain dose arginase suppresses eNOS activity, then supplements would be deemed counterproductive.

An arginine study in human subjects discovered that high dose supplementation of around 36 g/day of oral arginine accelerated the production of ornithine, a metabolite of the arginase pathway, whereas it did not have any significant effect on nitrate levels, which is a metabolite of the eNOS pathway (Beaumier et al., 1995). This suggests the possibility that L-Arg supplements may not be effective in increasing NO function with eNOS due to the arginase enzyme taking greater effect. Another study reported that inhibiting arginase stimulated the production of NO in endothelial

cells (Chicoine et al., 2004), which may suggest that arginase suppression may be necessary for the NO effects expected with L-Arg supplements in the body. More studies need to be accomplished to view a bigger picture on what really happens to these two major enzymes with oral arginine at various doses that are currently being taken by healthy people and those with disease conditions.

### 1.3.9 Enzyme Deficiency and its Consequences

Defects in the metabolic enzymes of the arginine pathways have been correlated with a variety of diseases, which is anticipated considering the important roles of metabolites of these enzymes in the body. A deficiency in liver arginase leads to hyperargininemia and to a lesser extent, excessive accumulation of ammonia in humans (Iyer et al., 1998). Hyperargininemia is associated with pathology in the central nervous system, including degeneration of the cortex and the pyramidal tracts causing neurological impairment, as well as growth retardation (Iyer et al., 2002).

eNOS deficiency on the other hand has been linked with hypertension, ischemic complications, and endothelial dysfunction in studies using knockout mice (Huang, 2000; Seinosuke and Mitsuhiro, 2004), which verifies the crucial functions of NO in the cardiovascular system. Under specific conditions, endothelial dysfunction was able to be reversed with arginine administration in both diabetic rats and hypercholesterolemic humans (Wu et al., 2009). ADMA is a naturally occurring endogenous inhibitor of NOS that suppresses NO production and leads to NO deficiency (Vallance et al., 1992). Elevated ADMA levels have been observed with several disease conditions including hypertension and kidney failure (Vallance et al., 1992). ADMA acts as a competitive inhibitor of NOS, thus increasing L-Arg concentration may be effective in reversing the inhibition (Böger, 2006).

ADC deficiency and its consequences have not been well studied yet, but considering the role of the ADC pathway, it could be anticipated that the deficiency may impair polyamine synthesis, which serve various roles in the body as discussed earlier.

AGAT deficiency is from a genetic mutation characterized by insufficient creatine levels in the brain, and low levels of its metabolites in the blood and urine, and it leads to myopathy, global developmental delay, and intellectual disability, which can be treated with oral supplements of creatine (Stockler-Ipsiroglu et al., 2015). This enzyme deficiency is recognized as one of the three types of inborn disorders causing cerebral creatine deficiency syndrome (Stöckler-Ipsiroglu, 1997).

For all cerebral creatine deficiency disorders, intellectual disability and developmental delay are common characteristics, whereas myopathy is a specific characteristic for just AGAT deficiency (Stockler-Ipsiroglu et al., 2015). A good news is that early detection and intervention for the condition can improve the neurologic symptoms and potentially allow for normal outcome after treatment (Battini et al., 2006; Ndika et al., 2012).

#### 1.3.10 Excretion

As mentioned earlier, arginine in systemic circulation is readily degraded and cleared from the body. A mammalian pharmacokinetic study of arginine (Wu et al., 2007) found that one-time intravenous administration of arginine temporarily increases the blood levels of arginine, before being brought back down to the baseline levels after just a few h. In this study, the effects of long-term administration of intravenous and oral arginine showed similar results, where for both routes of administration the serum levels of arginine were not significantly different in comparison to the control group, when blood samples were checked 5 h post-administration. A conclusion made from this finding is that dietary arginine is quickly metabolized and eliminated from the body, rather than accumulating in the circulation to be taken up later. The elimination half-life of oral arginine for adult humans is reported to be approximately 1 h (Bode-Böger et al., 1998).

The elimination of arginine does not usually take place through the kidneys because as with all amino acids, almost all of the filtered arginine gets reabsorbed back into the renal proximal tubules (Young and Freedman, 1971). Rather, arginine metabolites, such as nitrites, urea, and ornithine, formed after enzymatic destruction of arginine get eliminated out of the body through urine (Tangphao et al., 1999b). A human pharmacokinetic trial of arginine determined that approximately 5 g of L-Arg was eliminated in the urine when the subjects were infused with 30 g of intravenous L-Arg, whereas for an oral dose of 10 g, no excretion through the urine was even observed (Tangphao et al., 1999b). Direct excretion of intact arginine through the urine tends to occur when the renal reabsorption system is saturated with excess arginine in the tubules from high dose. For both oral and intravenous administration of arginine, L-Arg is eliminated out of the body in a biphasic pattern. In the first round there is a fast decline in plasma arginine levels due to renal clearance, followed by a more gradual fall in its concentration from non-renal excretion (Tangphao et al., 1999b).



## 1.4 Pharmacodynamics

### 1.4.1 Protein Expression and Activity Regulation

Interestingly, arginine affects its own metabolic fate by selectively regulating protein expression and activity of several enzymes, in a dose-dependent manner (Morris Jr, 2004). For instance, L-Arg represses the activities of arginosuccinate lyase and arginosuccinate synthetase, both of which take part in catalyzing arginine biosynthesis, though the mechanism of action for this molecular regulation has not been clarified (Schimke, 1962, 1964). Arginine also regulates the expression of iNOS, where overexpression of arginase or decreased arginine uptake causes insufficient extracellular L-Arg levels, which inhibit the expression of iNOS, thereby dysregulating the host defense system (Lee et al., 2003; Manner et al., 2003; Morris Jr, 2004). In addition, the level of arginine in the body affects expression of its transporters. CAT-1 expression is found to increase *via* increased transcription and translation of its gene, when there is restricted intake of amino acids such as arginine (Morris Jr, 2004).

### 1.4.2 Arginine and Growth Hormone Release

Arginine has been found to increase the release of growth hormone (GH) at a certain dosage in support of numerous oral and intravenous arginine supplementation studies. The mechanism of GH response augmentation by arginine is by downregulating the secretion of somatostatin, which is responsible for inhibiting the release of GH from the pituitary (Alba-Roth et al., 1988). By inhibiting the inhibitory factor for GH, arginine indirectly stimulates the release of GH and potentiates responsiveness to growth hormone releasing hormone (GHRH) (Collier et al., 2005). Studies that have tested the effects of intravenous arginine administration on GH response determined that both doses of 5 g and 9 g of arginine led to significantly elevated plasma GH levels in the resting state, whereas a higher dose of 13 g did not (Collier et al., 2005). It was further reported in this study that the 13 g dose was not tolerated well by most of the subjects, causing moderate gastrointestinal distress. However, several other studies that tested even a higher intravenous dose of 30 g reported the effectiveness of arginine in elevating GH concentrations (Weltman et al., 2000; Wideman et al., 2000; Kamel et al., 2002). The parenteral route of arginine administration has been shown to consistently cause increased plasma GH levels, whereas the enteral route has not (Chromiak and Antonio, 2002).

In contrast to the studies for intravenous administration of arginine, oral arginine supplementation studies reported conflicting results in regard to the physiological efficacy of arginine as a potent stimulatory agent for GH release. A low dose oral arginine study involving children demonstrated that 4 g of oral arginine in the form of arginine hydrochloride significantly increased both, the resting GH levels, and GH response to GHRH, released from the hypothalamus (Bellone et al., 1993). As well, another arginine study with healthy postmenopausal women given 9 g/day of oral L-Arg for 1 month suggested this dose to be safe and effective in increasing GH levels (Blum et al., 2000). On the other hand, some suggested that oral arginine supplements are likely not effective in increasing GH levels and discouraged taking it for this purpose, adding that it would likely cause unpleasant gastrointestinal symptoms such as stomach discomfort and diarrhea (Chromiak and Antonio, 2002). This conclusion was supported by other studies involving male weight trainers (Walberg-Rankin et al., 1994) and bodybuilders (Lambert et al., 1993), where they were provided oral arginine at a dose of 8 g/day for 17 days, and 2.4 g of arginine/lysine supplement dose, respectively. Neither of those studies showed significantly increased GH levels. With the variability in the study results, it is difficult to conclude the effect of oral doses of arginine supplements on GH release.

#### 1.4.3 Arginine and Endothelial Function

As outlined earlier, one of the widely known physiological functions of arginine is its effects on the vasculature to maintain good vessel health as a part of the eNOS pathway. As a substrate for eNOS and a precursor to NO, L-Arg is responsible for endothelium-dependent vasodilation, which is defective in individuals with endothelial dysfunction and many other cardiovascular diseases (Yang et al., 2009). A long-term arginine study involving patients with nonobstructive coronary artery disease reported that oral L-Arg taken three times a day for 6 months showed higher coronary blood flow in response to acetylcholine when compared with the control group, indicating improvement in endothelial function (Amir et al., 1998).

In addition to its role in vasorelaxation, NO also attenuates platelet aggregation (Radomski et al., 1990), and prevents adhesion of platelets to endothelial cells (Radomski et al., 1987). This contributes to the health of the endothelium as platelet interaction with the endothelium mediates cardiovascular issues such as vascular inflammation and atherosclerosis (Hamilos et al., 2018).

Endothelial dysfunction is highly associated with alteration in eNOS function with a subsequent decrease in NO levels and an increase in production of superoxide (Yang et al., 2009). The accumulation of superoxide anions causes oxidative stress in the endothelium and takes part in destroying NO, contributing to endothelial dysfunction (Vallance and Chan, 2001). Several studies have indicated that L-Arg exhibits antioxidant functions by acting as a free radical scavenger and increasing total antioxidant capacity in the body (Tripathi and Misra, 2009; Fazelian et al., 2014). All of these research reports complement the notion that L-Arg, in association with the product of its major metabolic pathway, *viz.* NO, plays a crucial role in maintaining normal endothelial function.

#### 1.4.4 Role of Arginine in Targeting Factors of Type 2 Diabetes

Arginine may be considered as a potential therapeutic agent for type 2 diabetes due to various physiological functions that have been demonstrated by multiple research studies. Firstly, many studies have demonstrated that arginine is effective in increasing insulin sensitivity in the body (Maccario et al.; Piatti et al., 2001; Lucotti et al., 2006; Bogdanski et al., 2012; Miczke et al., 2015). Insulin resistance, which is characterized by hyperglycemia, has been known to be closely linked with type 2 diabetes and currently is a target for the treatment of the disease (Taylor, 2012). Therefore, increasing sensitivity of insulin would allow for greater uptake of glucose into cells to ameliorate hyperglycemia and potentially reverse type 2 diabetes. In order to explain the mechanism of arginine action in alleviating insulin resistance, it has been speculated that insulin resistance is linked with reduced levels of cGMP, the second messenger of NO, and subsequent reduced vasodilatory response (Piatti et al., 2001). This proposal is supported by the conclusions drawn from other studies that insulin sensitivity is positively correlated with endothelium-dependent NO synthesis (Petrie et al., 1996), and that insulin resistant individuals show reduction in vasodilatory responses (Laakso et al., 1992). Thereby, L-Arg supplementation through the eNOS pathway, as described previously, increases endothelium-dependent NO production, which acts on guanyl cyclase to increase the levels of cGMP, allowing for more vasodilation, and increasing insulin sensitivity. Not only is arginine known to increase the sensitivity of insulin, but research studies have also suggested that arginine given intravenously and not orally directly acts as an insulin secretagogue by a mechanism associated with depolarization of the plasma membrane of islet B-cells (van Haeften et al., 1989; Sener et al., 2000).

In addition, an interesting and novel finding of both L- and D-Arg from a study is their ability to mitigate endothelial dysfunction caused by high levels of glucose and its metabolite, methylglyoxal (MG), both of which are highly associated with type 2 diabetes (Dhar et al., 2012). Rather than depending on the eNOS pathway, arginine directly acts as a scavenger for MG, which is a glycating compound that reacts with certain proteins to synthesize irreversible advanced glycation end products (AGEs) (Dhar et al., 2012). AGEs cause oxidative stress and vascular damage, leading to diabetic complications (Dhar et al., 2008). It has been found that the production of MG and MG-dependent AGEs are significantly elevated in diabetic patients, and this is also partly likely due to alteration in glyoxalase I, which is the enzyme responsible for degrading MG into D-lactate (Rabbani and Thornalley, 2014).

As arginine is capable of increasing insulin sensitivity and selectively targeting the mediator for vascular complications of diabetes *viz.* MG, arginine may potentially be effective in attenuating the pathogenesis of type 2 diabetes and its vascular complications.

#### 1.4.5 Arginine and Immune Function

Research on the physiological impact of arginine on immune function has been surrounded with controversies around its mechanism and treatment approach. The effects of arginine on immune function have been mostly associated with the production of its metabolites such as NO that serve as immunomodulators, as a part of the arginine metabolic pathways (Kim et al., 2018). While some studies suggest that arginine supplementation promotes immune responses to enhance defense mechanisms (Visek, 1986; Barbul et al., 1990; Sax, 1994), others have indicated the opposite, that arginine deprivation may help with immune defense and suppress cancer growth (Lind, 2004; Yoon et al., 2013).

There are numerous rationales that have been proposed to support both sides of the argument on arginine effects in immune function. First of all, iNOS plays a key role in mediating the function of immune cells as a part of the immune defense system. NO synthesized from iNOS acts as a proinflammatory agent in innate immune cells such as macrophages to destroy pathogens in the body (Bogdan et al., 2000). As a substrate for iNOS, L-Arg deficiency causes defects in the function and reduction in proliferation of T-cells, which was found to be recovered by arginine supplementation (Barbul, 1990; Rodriguez et al., 2007). This is because arginine plays a significant role in lymphocyte mitogenesis to improve immune function (Barbul et al., 1990), and thus

decreased bioavailability of arginine may mediate immune suppression. Arginine controls T-cell proliferation by directly facilitating the cell cycle of T-lymphocytes at the level of transcriptional and post-transcriptional regulation, acting as a thymotropic agent (Witte and Barbul, 2003). This conclusion has been supported by a study that demonstrated that a decrease in L-Arg availability causes cell cycle arrest at G<sub>0</sub> to G<sub>1</sub> phase for T-cells (Rodriguez et al., 2007). Also, speaking at the level of translation, arginine is responsible for regulating protein expression of an important subunit of the receptor complex for T-cells (Rodriguez et al., 2003; Popovic et al., 2007). Therefore, not only does arginine control proliferation of immune cells, but it also controls its action by modulating the formation of its receptors. Furthermore, as previously explained, L-Arg augments the release of GH, which increases maturation of myeloid progenitor cells, and the synthesis of lymphocytes and immunoglobulins (Meazza et al., 2004).

In contrast, excess arginine leading to excess production of the iNOS catalyzed metabolite, NO, has been suggested to promote growth and survival of cancer cells and mediate anti-cancer treatment resistance (Ekmekcioglu et al., 2017; Kim et al., 2018). Studies have reported that an increased NO production enhances immune suppression, and progression of tumors, which may be due to inhibitory action of NO on IL-12-mediated Th1 immune responses (Yoon et al., 2013; Wang et al., 2014b; Xue et al., 2018). In terms of NO function in macrophages, it can suppress its activation, leading to inflammatory diseases and infection (Xue et al., 2018). Interestingly in addition, it has been reported that arginine supplementation actually acts a source of arginine that melanoma cells depend on for survival, since a crucial enzyme for arginine synthesis is often missing in these cells (Yoon et al., 2013). Therefore, depleting free arginine in the body could be useful as a therapeutic approach to enhance immunity in fighting off these cells.

There is accumulating evidence for a variety of L-Arg mechanisms for both boosting immune function and downregulating immune response, thereby, the exact clinical benefit of arginine supplementation for disease therapy in patients is unclear to this day.

#### 1.4.6 Arginine and Wound Healing

Arginine through its metabolic pathways has been found to contribute to the wound healing process. There are many physiological differences between acute wounds and chronic wounds, and unfortunately, knowledge on the wound healing process has only been well established for acute and not chronic wounds (Mast and Schultz, 1996; Stechmiller et al., 2005). In short, normal

acute wound healing is characterized by three steps, in order: inflammation mediated by iNOS, cell proliferation in tissues, and remodeling, and each one of these three steps is strictly regulated by various factors including inflammatory cytokines (Stechmiller et al., 2005). Wound healing is promoted by arginine through the two of its major metabolic enzymes, NOS and arginase in sequential order (Abd-El-Aleem et al., 2000; Witte and Barbul, 2003; Stechmiller et al., 2005).

Firstly, arginine gets metabolized by all isoforms of NOS located in skin tissues such as keratinocytes and melanocytes (Stechmiller et al., 2005), synthesizing citrulline and NO as its metabolites as explained previously. NO contributes to wound healing by numerous mechanisms, but primarily by protecting the skin from cellular damage by causing vasodilation for better blood flow, inhibiting lymphocyte replication and killing pathogens (Albina and Henry, 1991; Mills, 2001; Nieves and Langkamp-Henken, 2002). The important isoform of NOS in wound healing is iNOS which is found mainly in macrophages, as this isoform produces NO in high bursts compared to the other two isozymes (Witte and Barbul, 2003). The increase in NO synthesis by NOS is followed by the suppression of NOS activity and a subsequent elevation in arginase activity to form increased levels of ornithine and proline (Stechmiller et al., 2005). Polyamines formed from ornithine by ODC regulate cell replication, cell growth and tissue regeneration, and proline formed from ornithine by OAT is a major collagen constituent that contributes to cell growth and the health of connective tissues (Stechmiller et al., 2005). Collagen is very important for the process of wound healing as it is responsible for scar formation (Witte and Barbul, 2003).

With results from numerous arginine studies focused on wound healing, it has become evident that arginine supplementation increases both hydroxyproline levels leading to a subsequent increase in collagen synthesis, and wound breaking strength (Seifter et al., 1978; Barbul et al., 1983, 1985; Shi et al., 2000, 2003, 2007; Wittmann et al., 2005).

## **1.5 Oral Arginine Supplements and its uses**

### **1.5.1 Optimal Oral Dosage**

In order to fully understand the safety and efficacy of oral arginine supplements, various factors must be considered, including the different target populations with certain conditions, dosage, and treatment duration. The safe range of oral doses for specific effects of arginine in the general population has not been clearly established yet. However, several animal and human clinical

research trials have contributed to knowledge on potential safe and effective dose ranges for arginine supplementation.

An animal study involving rats, pigs, and sheep concluded based on their results that human enteral arginine doses of 3, 10, and 15 g/day would be safe in a 5 kg infant, 30 kg child, and 70 kg adult, respectively (Wu et al., 2007). From the same study, it was also suggested that an enteral dose of arginine ranging from 15-40 g/day should be tolerable for 70 kg human adult.

There are many human clinical trials done to establish the safety and efficacy of oral arginine. One important study tested for the effects of dietary arginine at doses of 3, 9, 21, and 30 g/day for a full week in healthy subjects and found that 9 g/day was the dose where circulating L-Arg level in the blood was highest, with minimal side effects, appearing as the ideal dose (Evans et al., 2004). Other studies involving healthy adult volunteers reported that oral arginine supplementation was well tolerated with minimal adverse effects at a dose of 10 g/day (Tangphao et al., 1999b), and at an even higher dose of 40 g/day given for a full week (Beaumier et al., 1995).

Oral arginine supplements are believed to be safer and more effective in the body when taken in multiple lower doses rather than one high dose for a few reasons (Wu et al., 2007). One is that as mentioned previously, due to the fact that positively-charged amino acids share the same transporter for absorption, arginine at a significantly high dose can possibly alter the balance of other cationic amino acid levels in the body (Edmonds et al., 1987). Also, it has been noted that divided doses of arginine for administration helps with keeping the level of arginine in the blood elevated for longer (Böger and Bode-Böger, 2001), whereas a one-time arginine dose does not change the arginine levels in the blood when compared with baseline plasma concentration after a few h of administration (Wu et al., 2007). Prolonging the duration of arginine circulation in blood means that more of it can be thoroughly distributed across the body tissues to exert its effects. It is crucial to find the minimal effective dose, as high arginine doses may lead to detrimental effects. A high arginine dose causes rapid mass production of NO that may lead to atherosclerotic lesions (Chen et al., 2003) and several issues in the gastrointestinal tract (Wu and Meininger, 2000).

Further research is necessary to build a stronger foundation of understanding the exact effects of oral arginine at different doses and duration in the healthy population and those with disease conditions. So far, there is enough scientific evidence on many physiological functions and benefits of arginine, but practically limited evidence on the safety and efficacy of oral arginine supplements. Therefore, many individuals are relying on the theory behind arginine's NO

augmenting property in the body to take arginine supplements, rather than scientific evidence. Generally, oral arginine supplements are believed to be potentially effective for various uses like enhancing athletic performance, sexual function, and cardiovascular health (L-Arginine, 2021).

### 1.5.2 Use in Athletes

Healthy populations such as the athletes have been turning to L-Arg supplements for ergogenic purposes. A systematic review and meta-analysis that investigated the ergogenic potential of arginine supplements reported that both, one-time and chronic supplementation with oral arginine improved both aerobic and anaerobic performance if taken at doses of 0.15 g/kg at 60 to 90 min prior to exercise, and 1.5 to 2 g/day for 4 to 7 weeks, respectively (Viribay et al., 2020).

The physiological mechanisms behind the possible effectiveness of arginine supplements on physical performance involve the arginine metabolites NO and creatine, as well as the role of arginine in GH release (Campbell et al., 2004).

GH may be useful for building lean body mass and power through its anabolic properties (Saugy et al., 2006), and because arginine has been hypothesized to augment GH secretion in the body, it is commonly viewed as an ergogenic agent. For instance, a double-blinded study that supplemented 15 g/day of oral arginine-aspartate to endurance-trained runners for 2 weeks prior to a marathon run found that plasma GH levels were significantly elevated in these subjects, with blood samples collected before and after the full marathon run (Colombani et al., 1999). However, as explained earlier, studies regarding oral usage of arginine and its effects on GH secretion have reported conflicting results, suggesting the need for further investigation.

Creatine, one of the metabolites of the AGAT pathway of arginine, is well known in athletes for not just its role in regulating energy storage, which has been described earlier, but also for building muscle size and strength to increase exercise performance (Vandenberghe et al., 1997; Volek et al., 1999). Current evidence for the indirect benefits of oral arginine supplementation on physical performance related to creatine production have been associated with anaerobic or high-intensity exercise, such as weightlifting (Viribay et al., 2020). One of the main reasons for this is because rapid, high bursts of energy is associated with the phosphagen system, for which the main substrate is creatine (Viribay et al., 2020). Although arginine normally acts as a major substrate for AGAT to produce creatine in the body, oral arginine supplementation may be ineffective in causing elevation in creatine levels for ergogenic effects. This is because creatine supplementation



has been determined to suppress the AGAT enzyme (Walker, 1979), and the rate-determining step of creatine synthesis is regulated by AGAT and not arginine levels, for guanidinoacetate production (Stead et al., 2001). The better option would be to directly take creatine supplements.

NO made from arginine metabolism *via* the NOS pathway is known to serve important functions in physical performance. It causes vasodilation which helps with tissue respiration through enhancing oxygen uptake in muscles (Wolin et al., 1997). NO improves blood flow, making it easier for oxygen delivery and uptake into muscle tissues to increase tissue respiration (Wolin et al., 1997). With those functions, it is useful in reducing oxygen cost during exercise and improving exercise tolerance (Campbell et al., 2004; Jones, 2014). Thereby, evidence of benefit for arginine supplementation in endurance sports such as long distance running, has been considered to be linked to NO effects (Viribay et al., 2020). Although some research evidence on the benefits of NO in exercise has been established, the benefits of oral arginine supplements to augment NO production in the body to exert these beneficial effects, have not been thoroughly investigated yet in healthy individuals.

Overall, the available data on the impact of arginine supplementation on athletic performance remains controversial among various studies, and a consistently effective dose and duration for arginine supplementation has not been established yet either (Viribay et al., 2020).

### 1.5.3 Use in Erectile Dysfunction

Erectile Dysfunction affects over 50% of men in the age range of 40 to 70 years, with the number of people diagnosed with this condition increasing with age (Eardley, 2013). The main regulator for penile erection is NO formed from NOS, as the process of erection is regulated by vasorelaxation of smooth muscles in corpora cavernosa (Ignarro et al., 1987; Maggi et al., 2000). As L-Arg acts as the one and only substrate for NOS to produce NO in the body (Burnett, 2004), the role of arginine supplementation in potentially increasing physiological NO levels for the treatment of erectile dysfunction has been considered and studied. A study indicated that a common trend seen with patients with erectile dysfunction is the significantly reduced levels of L-Arg and L-citrulline, suggesting a link between this condition and the role of L-Arg (Barassi et al., 2017). Although phosphodiesterase inhibitors (PDE<sub>i</sub>) are first-line drugs for treatment of this condition, arginine supplements may prove to be an effective alternative. PDE<sub>i</sub> inhibit the breakdown of cGMP to cause relaxation in penile smooth muscle and maintain penile erection

(Rhim et al., 2019). Arginine supplements have been shown to cause less adverse effects and have better safety profile than PDE<sub>i</sub> (Rhim et al., 2019). As well, it also has been mentioned that as arginine supplements are more commonly viewed as nutrition supplements rather than drugs, it may be more psychologically accepted and easily considered for use than PDE<sub>i</sub> (Cormio et al., 2011). In terms of effectiveness of arginine supplements in men with erectile dysfunction, there are several studies that have demonstrated its efficacy at specific doses.

A prospective, randomized study tested for the efficacy and safety of L-Arg supplements at a dose of 2.5 g/day with and without 5 mg/day PDE<sub>i</sub> for 12 weeks, in patients with erectile dysfunction at various levels of severity (Gallo et al., 2020). Using the International Index of Erectile Function – Erectile Function Domain (IIEF-EF) in this study, L-Arg monotherapy was found to be significantly effective in patients with mild and moderate erectile dysfunction, but ineffective for people with the severe form of the disease. The effectiveness of arginine monotherapy was greatest for the mild severity population to the point that it was suggested to be equally effective as PDE<sub>i</sub> monotherapy with less side effects. Overall, combination therapy of L-Arg with PDE<sub>i</sub> showed greatest improvement for all patient populations in the study, including the ones with the severe form of erectile dysfunction. The conclusion made from this study was supported by others who reported similar results (Rhim et al., 2019; Abu El-Hamd and Hegazy, 2020; El-Wakeel et al., 2020). For instance, another erectile dysfunction clinical study in the elderly population using 5 g/day of arginine for 6 weeks with and without PDE<sub>i</sub>, also suggested combination therapy to be the outstanding method of treatment with best efficacy, though monotherapy was also shown to be effective (Abu El-Hamd and Hegazy, 2020).

#### 1.5.4 Use in Hypertension

Hypertension is known to be the leading contributor for both morbidity and mortality worldwide and acts as a major causal factor for cardiovascular diseases (Oparil et al., 2018). The impact of L-Arg supplementation has been linked with hypertension treatment, mainly due to the role of endothelium-dependent vasodilation associated with NO, increasing blood flow and decreasing vascular resistance (Khalaf et al., 2019; Gambardella et al., 2020).

In addition to vascular resistance and blood pressure, oxidative stress caused by reactive oxygen species also leads to vessel damage to contribute to cardiovascular disorders like hypertension and therefore, anti-oxidants play an important role in the maintenance of vascular health (Senoner and

Dichtl, 2019). A study done on mildly hypertensive individuals determined that 2 g of oral L-Arg three times a day for 28 days significantly increased the level of antioxidants to reduce oxidative stress (Jabecka et al., 2012).

Furthermore, several studies suggest that only specific subgroups may benefit from these supplements. Some indicated that arginine supplementation is effective specifically for salt-sensitive hypertension, rather than essential hypertension (Vasdev and Gill, 2008). As well, the majority of the benefits seen with L-Arg supplementation were in studies of hypercholesterolemic individuals, which showed that this population responded and benefited well in various cardiovascular aspects such as endothelium-dependent vasodilation and hypotensive responses (Clarkson et al., 1996; Theilmeier et al., 1997; Lekakis et al., 2002; West et al., 2005). This goes along with the findings that blood pressure regulation by oral arginine supplements is only effective for certain subgroups of unhealthy population such as people with diabetes or coronary artery disease, and not the healthy population (Ast et al., 2011; Dong et al., 2011; Martin and Desai, 2020). No significant effect on systolic and diastolic blood pressure was seen in healthy male subjects chronically taking oral L-Arg supplements at a dose of 2 g/day for 45 days (Pahlavani et al., 2014). However, there is also a diet-controlled human study which reported that oral L-Arg supplements at a dose of 10 g three times day for a week led to significantly lowered blood pressure in healthy volunteers, though the sample size of study was very small (Siani et al., 2000). Study results regarding the efficacy of oral arginine supplements in hypertension are not completely in consensus to conclude without a doubt that it is effective for the entire population. However, a significant trend of benefits has been observed for individuals with various cardiovascular diseases. More studies considering different factors such as the targeted dose, treatment duration, health status, comorbidities and baseline blood pressure would contribute to a better understanding of how effective oral arginine use would be in hypertension.

#### 1.5.5 Use in Hypercholesterolemia

High levels of cholesterol in the blood have been closely linked to cardiovascular complications such as atherosclerosis and coronary artery disease (CAD) (Kottke et al., 1988). Supplemental arginine use in hypercholesterolemia has been considered due to the theoretical knowledge of NO as a vasodilator and inhibitor of platelet aggregation (Moncada and Higgs, 1993). Clinical studies with hypercholesterolemic patients have determined that oral L-Arg supplementation at 6 to 10

g/day for 2 weeks and 8.4 g/day for 2 weeks, improves endothelial dysfunction (Maxwell et al., 2000) and platelet aggregation (Wolf et al., 1997), respectively, both of which are major characteristics of hypercholesterolemia. These two characteristics are very important to tackle to prevent the atherogenic process of hypercholesterolemia. As mentioned earlier, clinical studies of oral arginine effects on blood pressure mainly involved hypercholesterolemic patients. This may be due to the fact that cardiovascular diseases are often interconnected with one another, meaning that hypercholesterolemic patients likely have hypertension and the two conditions are both characterized by endothelial dysfunction. Endothelial dysfunction plays a major role in several cardiovascular disorders including hypertension, hyperlipidemia, and diabetes.

One study tested the effects of 7 g/day of oral L-Arg 3 times a day for 4 weeks in hypercholesterolemic individuals with endothelial dysfunction, by measuring the endothelium-dependent and -independent responses through brachial artery diameter (Clarkson et al., 1996). This with several other similar studies (Creager et al., 1992; Thorne et al., 1998) together presented that oral L-Arg supplementation leads to significant improvement in endothelium-dependent vasodilation in hypercholesterolemia. In addition, one study in particular also suggested that arginine supplementation efficacy is similar or even better than lipid-lowering therapies which are often used to reduce cholesterol levels in the blood (Stroes et al., 1995).

#### 1.5.6 Use in Type 2 Diabetes

Due to the possible roles of L-Arg as an antioxidant, insulin sensitizer and vasodilator, its use as an oral supplement in diabetes has been studied. Preclinical studies done using diabetic rats showed that oral arginine supplementation attenuates endothelial dysfunction, reduces oxidative stress and improves vasodilator responses (Pieper et al., 1996; Ozçelikay et al., 2000). As well, studies in both diabetic rat (Claybaugh et al., 2014) and human (Piatti et al., 2001) subjects found oral L-Arg supplementation to be effective in improving insulin sensitivity. Lastly, oral L-Arg supplementation at a dose of 6.4 g/day for 18 months in middle-aged individuals with defective glucose tolerance and metabolic syndrome lowered the risk of type 2 diabetes in the long term, measured by the cumulative incidence in a 90-month follow up period (Monti et al., 2018), suggesting its potential as a preventive. This suggestion is also supported by the fact that both L- and D-Arg directly act as a scavenger for MG, a major contributor to AGEs that is known to cause vascular complications of diabetes (Dhar et al., 2012). However, in contrast, there is a study that

demonstrated that L-Arg, in the process of interacting with MG and inhibiting AGEs formation, ends up producing superoxide anions as a byproduct (Tsai et al., 2004). With this result, the researchers of this study concluded that it may be counterintuitive to use L-Arg supplementation for prophylaxis in diabetic patients, as the harmful product can worsen oxidative stress that already exists in these patients. In conclusion for now, the evidence for the clinical use of oral arginine supplements in diabetic population overall is controversial and unclear (Tousoulis et al., 2002).

#### 1.5.7 Use in Heart Failure

The physiological roles of L-Arg have been associated with alleviating heart failure by contributing to NO formation, which causes vasodilation to reduce preload and oxygen demand on the heart (Macdonald et al., 1996; Le Corvoisier et al., 2000). The benefits of oral arginine treatment on heart failure were mainly reported for chronic, and not acute supplementation. A randomized, double-blinded, placebo-controlled arginine study testing for oral doses of 5.6 to 12.6 g/day for 6 weeks reported that the oral arginine supplementation improves blood flow, functional status in terms of walking distance, and arterial compliance, in heart failure patients (Rector et al., 1996). Another study determined that chronic oral arginine supplementation may be beneficial to improve daily functioning, according to the results indicating that chronic but not acute supplementation, at 6 g twice a day for 6 weeks, delayed ventilatory threshold (Doutreleau et al., 2005). As with other cardiovascular diseases, endothelial dysfunction is a key player in heart failure (Chin-Dusting et al., 1996), confirming the notion that NO serves important roles for this condition. In contrast to various studies that evidently suggested the efficacy of oral L-Arg supplements on heart failure, a study of heart failure patients taking 20 g/day of oral L-Arg supplements for 28 days demonstrated that in these patients, hypotensive responses were not significantly affected (Chin-Dusting et al., 1996). Thus, it was concluded from this study that oral arginine supplementation does not lead to improvement in endothelial function in heart failure patients. In order to better understand the therapeutic potential of oral arginine supplementation in heart failure patients, further clinical trials for both acute and chronic supplementation are needed.

#### 1.5.8 Use in MELAS Syndrome

Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome is a multi-organ disorder characterized by sudden, short-lived, and recurring occurrences

of stroke-like episodes (Ikawa et al., 2020). NO deficiency and endothelial dysfunction have been demonstrated to be closely associated with the MELAS syndrome, especially in the stroke-like episodes (Koga et al., 2005; El-Hattab et al., 2015). L-Arg therapy is commonly used for treatment of MELAS patients, and the mechanism has been linked with increased blood flow from increased NO levels (El-Hattab et al., 2017).

A study involving MELAS patients treated with oral L-Arg supplements at a dose of 0.15 to 0.3 g/kg/day for 18 months reported that the patients had elevated baseline ADMA levels, suggesting subsequent downregulation of eNOS to be at play in MELAS (Koga et al., 2005). The same study also found that the oral supplementation improved clinical symptoms of MELAS, reducing both the frequency and severity of stroke-like episodes and did not cause any safety concerns associated with side effects. Similarly in a recent long-term study involving people with MELAS symptoms treated with oral doses of 0.3 to 0.5 g/kg/day in three divided doses following each meal for two years, a tendency towards improvement in incidence and severity of ictuses (strokes) was observed, though not statistically significant (Koga et al., 2018).

Currently, oral arginine supplementation is being used commonly as prophylaxis to reduce recurrent stroke-like episodes, whereas the intravenous route is reserved for acute use, such as during a stroke-like episode (El-Hattab et al., 2017). In specific terms, the recommendations for the dose of oral arginine for prophylaxis is 150 to 300 mg/kg/day in 3 divided doses (Sproule and Kaufmann, 2008; Koenig et al., 2016).

A major limitation is that clinical studies of L-Arg supplementation on MELAS only address and focus on the stroke-like episodes of this disease characterized by many other factors. Therefore, more arginine studies covering and assessing the other aspects would allow for a bigger picture in understanding the therapeutic effects of oral arginine in this disease.

## **1.6 RATIONALE OF STUDY**

Oral arginine supplements are widely being used by both healthy and unhealthy populations for its physiological functions and presumed effectiveness for various conditions. However, these supplements are being used mostly according to the theoretical knowledge on the impact of arginine in the body, rather than reliable scientific facts proven through clinical studies. There still is a huge gap of evidence and knowledge on safety and efficacy of oral arginine supplements, and the recommended doses for use in particular conditions are needed to be further investigated. My

focus for this present study was on the effects of chronically administered oral arginine on arginase, eNOS, AGAT, and ADC pathways, and this study was extended from a lower dose Sprague-Dawley (SD) rat study of 500 mg/kg/day at 4-week (Martin and Desai, 2020), that has been published in our lab with some interesting and significant findings. The experimental protocol for my research is comparable to the lower dose study, but the main differences are the dose and duration of treatment of the rats. For this research study, oral arginine was administered for 16 weeks at a dose of 1000 mg/kg/day, so the arginine effects were examined from rats treated with twice the dose and four times the treatment duration in comparison to the previous study.

Along with many other D-isomers of amino acids, D-Arg is commonly known to be physiologically inert and thus is left out from consideration in most arginine studies. In order to verify this, we incorporated testing for the effects of oral D-Arg supplementation along with the L-isofom on the metabolic pathways of SD rats. From the lower dose study of 500 mg/kg/day for 4-week treatment mentioned previously, there were some unanticipated results of D-Arg appearing to exert significant effects on the levels of some metabolites and enzyme expressions from the four arginine pathways. Thereby, the results from this chronic higher dose study were aimed to be compared with the results of the lower dose study to observe the trend and understand the overall picture of the effects of both L- and D-Arg supplementation on the arginine metabolic pathways.

Oral arginine supplements are not only commonly used by individuals with unhealthy conditions such as cardiovascular diseases, but also by healthy individuals such as athletes for ergogenic purposes. As mentioned previously, current evidence on beneficial effects of arginine supplements is limited, and more research needs to be done on the general population to determine how and at what dose oral arginine can cause beneficial and harmful effects on the body of normal and healthy individuals. SD rats were used as standard, normal models for this study to test for the metabolic effects of supplemental arginine in healthy bodies and to establish a basis for understanding safety and efficacy of chronic supplementation of oral arginine in the general population as a contributor for pre-clinical study. On top of this present study, there is another separate study in progress in our lab using Zucker Diabetic Fatty rats as a pathological model, to understand the physiological roles of oral arginine in the unhealthy, diseased population.

## 1.7 HYPOTHESIS AND OBJECTIVES

### 1.7.1 Hypothesis

**Administration of oral L-arginine (1000 mg/kg/day) to 9-week-old male Sprague-Dawley rats for 16 weeks will lead to changes in enzyme expressions and activities, and/or the levels of metabolites, of arginine metabolic pathways, whereas administration of oral D-arginine (1000 mg/kg/day) for 16 weeks will not affect the arginine metabolic pathways.**

### 1.7.2 Objectives

1. To determine the physiological impact of a dose of 1000 mg/kg/day of oral arginine administered for 16 weeks, on its metabolic pathways in male Sprague-Dawley rats and compare it with the effects of 500 mg/kg/day of oral arginine administered for 4 weeks and published from our lab (Martin and Desai, 2020).
2. To determine the expression of L-arginine metabolizing enzymes, *viz.* arginase, eNOS, AGAT and ADC/agmatinase, and their activities, whenever possible, after treatment with L-Arg or D-Arg at 1000 mg/kg/day for 16 weeks in male Sprague-Dawley rats.
3. To determine the levels of metabolites of L-arginine metabolizing enzymes, *viz.* arginase, eNOS, AGAT and ADC/agmatinase, after treatment with L-Arg or D-Arg at 1000 mg/kg/day for 16 weeks in male Sprague-Dawley rats. The metabolites measured include urea, hydroxyproline, nitrite, creatinine and total polyamines. The metabolites chosen for measurements were based on the availability of their respective assay kits. Thus, creatinine was measured instead of creatine, hydroxyproline instead of proline and total polyamines instead of individual polyamines such as putrescine, spermine and spermidine.
4. To determine the effects of D-Arg, a supposedly physiologically inert isomer of arginine, at 1000 mg/kg/day for 16 weeks in male Sprague-Dawley rats, and compare it to the effects of L-Arg.
5. To measure the levels of lysine, which uses the same transporter as arginine, *viz.* CAT-1, in order to determine whether oral arginine affects lysine levels and availability.
6. To determine the effects of oral arginine on the levels of ADMA, a product of protein methylation post-translational modification, because ADMA is associated with homocysteine levels, cardiovascular diseases and may be affected by oral arginine supplements (Bode-Böger et al., 2003; Krzyzanowska et al., 2006)



## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Animals

All animal protocols for this study adhered to the guidelines of the Canadian Council on Animal Care and had received ethical approval by the Animal Care Committee at the University of Saskatchewan (Animal Care Protocol #20160059).

The animals used for this research project were eighteen 9-week-old, male Sprague-Dawley rats purchased from Charles River Laboratories in Quebec, Canada. The rats were fed a standard diet of a laboratory rat (Prolab® RMH 3000, LabDiet) provided by the Animal Care facilities. The standard diet which contained 22.5 % of protein in total, consisted of 1.41 % arginine.

Following acclimatization for 1 week, the rats were randomly divided into the following treatment groups:

- (1) **Control group:** Normal diet + **plain drinking water** (ad libitum) ( $n = 4$ ).
- (2) **L-Arg group:** Normal diet + **L-arginine (1000 mg/kg/day)** in drinking water ( $n = 7$ ).
- (3) **D-Arg group:** Normal diet + **D-arginine (1000 mg/kg/day)** in drinking water ( $n = 7$ ).

The treatment was continued for 16 weeks. During the entire treatment period of 16 weeks, each rat was placed alone in a separate cage for the recording of individual daily intake of water.

To prepare arginine stock solution, the arginine free base (L-Arg, Cat # W381918, Sigma-Aldrich Canada Ltd; D-Arg, Cat # GM7267, Glentham Life Sciences, Corsham, U.K.) was dissolved in the drinking water, and the highly alkaline pH was adjusted to 7.4 with hydrochloric acid. The reasons for the method of administering arginine in drinking water for the treatment groups, rather than through oral gavage or parenteral administration, were because it reflects the route that humans take when taking oral supplements, and daily oral gavage for 16 weeks was considered to be extremely stressful with an increased risk of mortality for the rats, due to the possibility of pulmonary aspiration. A higher dose of 1000 mg/kg/day administered for a longer period of 16 weeks was chosen for my project, in order to compare its physiological impact with a smaller dose of 500 mg/kg/day administered for a shorter duration of 4 weeks that was used in a study published by our lab recently (Martin and Desai, 2020). In terms of human equivalent dose, the dose of 1000 mg/kg/day used in my study is equivalent to a dose of about 162 mg/kg/day for humans (approx. 11.3 g for a 70 kg adult), according to the conversion guide provided by the Food and Drug Administration (2005). This dose can be considered to be moderate for humans because a meta-analysis that included 11 trials studying the effect of arginine supplements on blood

pressure in humans used arginine doses ranging from 4 to 24 g/day for periods of 2 to 24 weeks (Dong JY et al. 2011).

The body weights of all the rats were recorded prior to and after the full treatment period, and every other day during the treatment period. The water intake for each rat was recorded every other day. To account for the variation in daily water intake and the gradually increasing body weights, and to ensure that all rats get the same dose through the drinking water, the dose of arginine to be added to the drinking water for each rat was calculated with a specially devised formula:

**Body weight (g) x 15 / water intake per day (mL) = X mL of 10 g/L stock L-Arg solution to be added to a 300 mL bottle and the rest tap water to make 300 mL.**

Arginine was supplemented in the drinking water every other day based on calculations for that day. After the end of the 16-week treatment, the rats in all three groups were individually placed in metabolic cages which were used to collect urine over a 16 h period of overnight fasting with free access to water, but not food. The amount of urine collected in metabolic cages varied a lot from rat to rat, ranging from 0.3 mL to 55 mL, which made the urinary measurements of various metabolites unreliable. Thereby, urinary arginine levels were not measured. The metabolic cages were not connected to any instruments for metabolic measurements. The rats were anesthetized, and the blood samples were collected. After centrifugation of the blood samples at 12,000 rpm for 10 min, plasma was collected, frozen and stored at -80°C for further analysis. Finally, the rats were euthanized by cutting the heart open for exsanguination. Following euthanasia, organs and tissues were removed, rinsed with phosphate-buffered saline, frozen in liquid nitrogen, and stored in the -80°C freezer.

## 2.2 Homogenization of Tissues

Using a mortar and pestle, various organs including the aorta, liver, ileum, kidneys, brain, lungs and skeletal muscle were ground into fine powder under liquid nitrogen. The powdered samples were stored in cryogenic tubes at -80° C. To prepare tissue lysates, about 15 mg of powdered tissue was taken in a 1.5 mL Eppendorf tube and vortex mixed with the homogenization buffer, composed of 1% protease inhibitor cocktail (Cat. # P8340 Sigma-Aldrich) in 1 M Tris + 0.5 M EDTA + 0.3 M sucrose buffer. The protease cocktail contained, AEBSF [4-(2-aminoethyl)

benzenesulfonyl fluoride hydrochloride, 104 mM], aprotinin (80  $\mu$ M), bestatin (4 mM), E-64 (1.4 mM), pepstatin A (1.5 mM) and leupeptin (2 mM). The tissue mix was sonicated (3x10 sec pulses) using a polytron ultrasonic homogenizer. Following homogenization, the samples in the tubes were centrifuged for 10 min at 14000rpm to collect the supernatant from each tube. The majority of this supernatant collected was stored at -80°C for later use in various assays, whereas the remaining was used to prepare for the western blot homogenates described below.

### 2.3 Western Blotting

Western blot experiments were performed in cell lysates of tissue samples to determine the enzyme expression in the treatment groups in comparison to the control group. Firstly, western blot homogenates were prepared as follows: the homogenates were mixed with 4x Laemmli sample buffer (Cat# 161-0737, Bio-Rad Laboratories) and 2.5% 2-Mercaptoethanol (Cat# M6250, Sigma-Aldrich) in each tube, before being denatured at 95°C for 5 min in a digital heat block (Fisher Scientific, Ottawa, ON, Canada). In order to measure the protein concentration of the homogenates in each tube, protein assay was completed with using the Detergent Compatible Protein Assay (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

The loading volume of homogenates was determined by trial and error of loading amount between 30  $\mu$ g and 75  $\mu$ g, observing which one gives the best signal for the imaging software. The samples were loaded into 4-20% SDS-polyacrylamide pre-cast gels (Cat. # 456-1094, Bio-Rad Laboratories Ltd.), where each gel contains 10 of 50  $\mu$ L wells. The first well was always loaded with 5  $\mu$ L of Precision Plus Dual Color Standards (Cat. # 161-0374, Bio-Rad Laboratories Ltd.), to make sure that the bands detected are the proteins of interest at a specific molecular weight. The sample proteins in the gels were separated by electrophoresis, in an electrophoresis tank filled with electrode running buffer, prepared with ingredients from Bio-Rad Laboratories Ltd., Mississauga, ON, Canada: tris-Base (15 g/L, Cat. # 161-0719), glycine (72 g/L, Cat. # 161-0724), and sodium dodecylsulfate (SDS 15 g/L, Cat. # 161-0302). The gels were run at 50V initially for 5 min, followed by 100V for 1 hour and 25 min.

Following electrophoresis, the separated protein bands on the gel were wet electro-transferred at 100V for 3 h at 4°C to a 0.45 $\mu$ m polyvinylidene difluoride (PVDF) membrane (Cat. # 45004110, GE Healthcare Life Sciences, Mississauga, ON, Canada). The transfer occurred in a tank filled

with transfer buffer, made of tris-Base (5.85 g/L), glycine (2.93 g/L), SDS (0.373 g/L), and 20% Methanol (Cat. # A452-4, Fischer Scientific, Ottawa, ON, Canada), with pH adjusted to 9.2.

After the transfer, the membrane was blocked at room temperature with 5% bovine serum albumin (BSA, Cat. # A7906, Sigma-Aldrich Canada) on a shaker for an hour, in order to prevent non-specific binding of antibodies. This was followed by primary antibody incubation on a rocker at 4°C overnight (12 to 16 h). In order to dilute the antibodies prior to incubation, tris-buffered saline with tween (TBST) was prepared, with the following ingredients: Tris-Base (2.423 g/L), Sodium Chloride (NaCl, 8.766 g/L, Cat. # S271-3, Fischer Scientific), and 0.1% Tween-20 (Cat. # BP337-100, Fischer Scientific Canada, Ottawa, ON Canada). As with TBST, 1% BSA was also added when diluting the antibodies accordingly: eNOS (1:500, Cat. # 611852, BD Transduction Laboratories, Mississauga, ON, Canada), SLC7A1 (CAT-1, 1:1000, Cat. # ABIN5965961, Antibodies-Online Inc., Atlanta, GA, USA), arginase I and arginase II (1:1000, Cat. # ab91279 and Cat. # ab203071, respectively), ADC (1:1000, Cat. # ab157214), agmatinase (1:1000, Cat. # ab231894), GATM (1:1000, Cat. # ab87062), and the loading control,  $\beta$ -actin (1:1000, Cat. # ab16039), all purchased from Abcam Inc., Toronto, ON, Canada.

Following overnight incubation, the membrane was washed thoroughly with TBST five times at 5 min per wash, for a total of 25 min. After the wash, the membrane was subsequently incubated with either anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Anti-mouse, Cat. # 1706576, and Anti-rabbit, Cat. # 1706515, Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) for an hour at room temperature on a shaker. The secondary antibody was diluted at 1:10000 with TBST and 5% BSA. Following the secondary incubation, the membrane was again washed thoroughly for 25 min, as previously described. Finally, Clarity Western Enhanced Chemiluminescence Blotting Substrate (Cat. # 1705061, Bio-Rad Laboratories Ltd.) was applied on the membrane and incubated for about 30 sec before imaging on the ChemiDoc Imaging System (G:BOX Chemi XX6, Syngene, Frederick, MD, USA). The images of the protein bands were manually quantified using GeneTools software (Syngene, Frederick, MD, USA).

For loading control of western blotting data, the Invitrogen No-Stain Protein Labelling Reagent (Cat#A44449, Fisher Scientific) was used to perform total protein normalization. This reagent allowed for simple and quick visualization and quantification of all the proteins on the transferred membranes. Following the wet electro-transfer, each membrane was washed twice at 2 min each on a shaker, with ultrapure water. Then, 10 mL of No-Stain Membrane Labelling Solution was

added to the plate with the washed membrane and put on a shaker for 10 min for labelling reaction to occur. This was followed by another subsequent set of wash, three times at 2 min each. Finally, the membranes were imaged using the ChemiDoc Imaging System for visualization of the protein bands on the membranes.

## 2.4 Arginine Assay

The levels of arginine in the samples of plasma, liver, kidney, brain, ileum, skeletal muscle, aorta, and lungs were measured using a fluorometric Arginine Assay Kit (Cat. # ab252892, Abcam Inc., Toronto, ON, Canada). This is a quick and specific enzyme-based assay, where L-Arg metabolites create a detectable fluorescence signal after reacting with a probe. The samples used for this assay were filtered using an ultrafiltration device with a 10 kD cut-off Spin Column (Cat. # MRCPRT010, Millipore Sigma, Oakville, ON, Canada). In brief, 250  $\mu$ L for each of the sample supernatant prepared and stored at  $-20^{\circ}\text{C}$ , as described above, was very gently transferred to the 10 kD Spin Column using a pipette. After making sure that the caps are tightly closed, the columns with the samples were centrifuged at  $10000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . Subsequently, the filtrate was collected in a separate tube to be used for the assay. For each test sample, there were three wells assigned in the microplate, one background well, one spike well, and one sample well. The background noise readings are there to get subtracted from the test sample and spike readings. Spike is referred to as the internal standard that is used to rule out the matrix effect in the samples. The reaction mix and background mix were separately prepared in this assay. The reaction mix was added to the blank, standard, sample, and spike wells, whereas the background mix was added to the just the background wells. Following the addition of the mixture, the plate was incubated protected from light, for an hour in a warm water bath with temperature controlled at  $37^{\circ}\text{C}$ . The fluorescence was measured with a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo-Fisher Scientific, Vantaa, Finland) at 535/587 nm excitation/emission wavelengths.

## 2.5 Arginase Activity Assay

Arginase activity was quantified in samples of plasma, ileum, liver, and kidney, using the colorimetric Arginase Activity Assay Kit (Cat. # ab180877, Abcam Inc., Toronto, ON, Canada). This is an assay for kinetic mode detection, to quantify the activity of arginase. Since excess urea in samples can interfere with the assay, the samples were filtered using a 10 kD cut-off spin column,

prior to performing the assay. The samples, standards and controls were first loaded in a 96-well microplate. The background control mix was added to the background wells, and the substrate mix was added to each sample and positive control well and incubated for 20 min in a water bath at 37°C. Subsequently, the reaction mix was added to all the standard, sample, control wells. This is where arginine intermediates produced from arginase in the samples were reacted with a probe to develop a coloured product. Immediately after the addition of the reaction mix, the optical density of the product was read at 570 nm in a kinetic mode for 30 min using a microplate Spectrophotometer (Multiskan Spectrum, Thermo-Fisher Scientific, Vantaa, Finland).

## 2.6 Urea Assay

Urea is a product of arginase catalyzed breakdown of L-Arg. Urea levels in plasma, urine, liver, kidney, ileum, brain, skeletal muscle, and lungs were measured with a colorimetric Urea Assay Kit (Cat. # ab83362, Abcam Inc., Toronto, ON, Canada). This assay kit allows for quick and reliable detection by forming a product from urea that subsequently reacts with a probe to develop a gradation of colour in a microplate. The brain and lung samples were diluted 1:10 by adding 5 µL of sample to 45 µL of the provided assay buffer. For kidney, the samples were diluted 1:20 by adding 5 µL of sample to 95 µL of assay buffer. The rest of the samples including plasma and urine were used without additional dilution. In the sample and background wells in the 96-well microplate, 10 µL of each of the diluted samples were carefully loaded. The volumes in the sample and background wells were adjusted to 50 µL per well with the assay buffer, and 50 µL of each of the standard solutions was directly loaded in the standard wells. Afterwards, 50 µL of the reaction mix was then added into the each standard and test sample well and 50 µL of the background mix was added into the background wells, making the total volume in all wells 100 µL. Following the mix, the plate was incubated in a 37°C water bath with the lid closed to protect from light, for an hour. Finally, after incubation, the optical density was read at 570 nm in a spectrophotometer (Multiskan Spectrum, Thermo-Fisher Scientific, Vantaa, Finland).

## 2.7 Hydroxyproline Assay

Proline and hydroxyproline are derived from L-glutamate, which in turn is metabolically related to L-ornithine formation from L-Arg catalyzed by arginase. Hydroxyproline levels in the samples of plasma, liver, ileum, and kidney were measured using the colorimetric Hydroxyproline Assay

Kit (Cat. # ab222941, Abcam Inc., Toronto, ON, Canada). In this assay, hydroxyproline in the samples was first oxidized into a pyrrole intermediate. The oxidation reagent mix was added to each well in the microplate containing samples and standards, and the plate was incubated for 20 min at room temperature. Subsequently, the pyrrole intermediate formed was further reacted with an acidic developer reagent containing perchloric acid, which is a toxic and very reactive substance, handled and disposed carefully according to the protocol. After incubation with oxidation reagent mix, a developer was added and incubated in a warm water bath at 37°C, for 5 min. Right after, 4-dimethylamino benzaldehyde (DMAB) concentrate was added and incubated using a heat block at 65°C for 45 min. In result, a coloured chromophore was ultimately generated and its absorbance was measured using a microplate Spectrophotometer (Multiskan Spectrum, Thermo-Fisher Scientific, Vantaa, Finland) at 560 nm.

## 2.8 Nitric Oxide Synthase Activity Assay

NOS activity was quantified in the aorta, liver, ileum, kidney, brain, lungs, and skeletal muscle, using the colorimetric Nitric Oxide Synthase Activity Assay Kit (ab211083, Abcam Inc., Toronto, ON, Canada). This assay kit detects activity of all isoforms of NOS, endothelial NOS, neuronal NOS, and inducible NOS. In this assay, the sample homogenates were added with a reaction mix that consists of L-Arg content to be served as a substrate to the NOS enzyme. The mixture was incubated for 1 hour in the water bath at 37°C. This was followed by the addition of NOS Assay Buffer and enhancer solution, which was incubated for 10 min at room temperature. Then, Griess Reagents 1 and 2 were added and mixed in the plate wells and incubated for 10 min at room temperature to develop a colour. As mentioned previously for the nitrate and nitrite assay, the Griess Reagents are responsible for converting nitrate into nitrite, followed by conversion of nitrite into a purple azo product. Immediately after the 10-minute incubation period, absorbance at 540 nm was read for all wells in a plate reader.

## 2.9 Nitrate/Nitrite Assay

Nitrate and nitrite levels in plasma, liver, ileum, kidney, brain, skeletal muscle, aorta, and lungs were measured using the Nitrate/Nitrite Colorimetric Assay Kit (Cat. # 780001, Cayman Chemical, Ann Arbor, MI, USA). This assay used the samples that were filtered using the 10 kD cut-off Spin Column. The final products of NO after reacting with various molecules in the body, are nitrate

and nitrite, and since the proportion of the two metabolites are variable, the sum of levels of both metabolites are determined as an indicator for total NO production. This assay is based on Griess reaction, employing a two-step process. Nitrate in the sample is first converted into nitrite by the enzyme, nitrate reductase, and subsequently using Griess reagents, nitrite is converted into an azo chromophore, appearing dark purple in colour. After all the samples and standards were added into the microplate, enzyme cofactor and nitrate reductase mixtures were added and incubated for three h at room temperature. After the incubation period, Griess reagent 1 was added to all the wells, immediately followed by Griess reagent 2. Lastly, the plate was left for 10 min at room temperature for the development of colour, before the optical density of the final azo product was measured on a microplate Spectrophotometer (Multiskan Spectrum, Thermo-Fisher Scientific, Vantaa, Finland) at 540nm to detect the nitrite concentration.

## 2.10 Creatinine Assay

Creatine is a product of catalysis of L-Arg by AGAT. Creatine is converted to creatinine. Creatinine levels in the samples of plasma, urine, liver, kidney, ileum, brain, and skeletal muscle, were measured using a colorimetric Creatinine Assay Kit (Cat. # ab65340, Abcam Inc., Toronto, ON, Canada). In order to prevent errors in the assay from excessive protein concentration, samples were deproteinized using a 10 kD cut-off Spin Column prior to beginning the assay. In this assay, a probe reacts with a metabolite of creatinine produced from a series of reactions to develop a colour. To briefly outline the enzymatic reactions that occurred in this assay, creatinine in the samples first got converted into creatine by the enzyme, creatininase. Subsequently, creatine was converted into sarcosine by the enzyme, creatinase. Following an oxidation reaction of sarcosine led to the synthesis of a product that yields a red-coloured product. The optical density of this final, coloured product was measured at 570 nm on a microplate Spectrophotometer (Multiskan Spectrum, Thermo-Fisher Scientific, Vantaa, Finland), after the addition of the reaction mix and background mix in the corresponding wells as described above, and subsequent incubation for an hour in a water bath kept at 37°C.

## 2.11 Total Polyamine Assay

Polyamines such as spermine and spermidine are partly derived from putrescine, another polyamine, which in turn is a metabolic product of L-Arg catalyzed by ADC and agmatinase. Total



polyamine levels were measured in samples of plasma, liver, ileum, kidney, and brain using the fluorometric Total Polyamine Assay Kit (Cat. # ab239728, Abcam Inc., Toronto, ON, Canada). This assay used the samples that were filtered using a 10 kD cut-off Spin Columns. In this assay, the polyamines in the samples first reacted with an enzyme reaction mix to synthesize hydrogen peroxide. Hydrogen peroxide was then reacted with a polyamine probe to develop a fluorescence signal. The probe was diluted 1:10 right before use, with anhydrous dimethyl sulfoxide (DMSO). The plate was incubated in the water bath, in the absence of light for 30 min at 37°C. The fluorescence of the final product was measured with a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo-Fisher Scientific, Vantaa, Finland) at 535/587 nm excitation/emission wavelengths.

### 2.12 Lysine Assay

Lysine levels in the samples of plasma, liver, ileum, and kidney were quantified by a fluorometric Lysine Assay Kit (Cat. # ab273311, Abcam Inc., Toronto, ON, Canada). Similar to the arginine assay kit, the lysine assay is also based on enzymatic metabolism, where a detectable fluorophore is produced after intermediates of lysine react with a probe. As well, the samples were deproteinized using a 10 kD Spin Column to prevent potential interference with enzymes found in the samples. The protocol of this assay is very similar to the arginine assay, in that for each sample, there were three parallel wells, one for sample, one for spiked sample, and one for background control. Additionally, to the spike wells were added 4 µL of the lysine standard solution. After the addition of samples and the standard solution in the wells of the plate, the volume of all the wells was adjusted to 60 µL with the provided assay buffer. As with the arginine assay, the reaction mix was added to all test sample and spike wells, and the background mix was added just to the background wells. This was followed by an incubation period of 45 min in the absence of light at 25°C. Finally, the fluorescence was measured with a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo-Fisher Scientific, Vantaa, Finland) at 535/587 nm excitation/emission wavelengths.

### 2.13 Asymmetric Dimethylarginine Assay

Asymmetric dimethylarginine (ADMA) is produced by methylation of proteins and it is believed to compete with L-Arg for NOSs. ADMA levels in the plasma, ileum, liver, and kidney

were measured using the colorimetric Asymmetric dimethylarginine ELISA Kit (Cat. # OKEH02587, Aviva Systems Biology, San Diego, CA, USA). The provided microtiter well-plate in this kit is pre-coated with antibody against ADMA. Firstly, the standards and samples were added into the wells of pre-coated anti-ADMA microplate. Immediately after ADMA-Biotin Complex was added in all the wells except the blank and incubated for an hour at room temperature. As there are limited anti-ADMA coated in the well-plate, the biotinylated ADMA competes with ADMA in the samples. The plate was washed three times with the provided wash buffer with 2 min contact time each to remove any unbound ADMA. The washed wells were incubated with the Avidin-HRP Conjugate, for 45 min at room temperature. Following incubation, the liquid was disposed and thoroughly washed again in the same way as described previously. A detection substrate, TMB substrate, was added to the wells and incubated in the water bath at 37°C protected from the light for around 10 to 15 min. The gradations of the blue colour in the standard wells were checked every 3 to 5 min, to stop the reaction at an optimal colour. After observing an optimal development of colour, the stop solution was finally added to each well, where immediately with this addition, the colours changed to yellow gradations. Within 5 min of adding the stop solution, absorbance was measured at 450 nm using a microplate Spectrophotometer (Multiskan Spectrum, Thermo-Fisher Scientific, Vantaa, Finland).

## 2.14 Statistical Analysis

A power analysis was completed in the previous study in our lab (Martin and Desai, 2020), using G\*Power (v 3.1.9.4), to choose the sample size. We used at least four different parameters measured (significant and non-significant) and with a  $P=0.05$  and a power of 0.80, which gave us actual power values above 0.95 and a total sample size ranging from 6 to 15, which justifies our total sample size of 18 (4+7+7). Statistical analysis of the western blotting and assay results was performed using Graphpad PRISM software version 8, through one-way ANOVA with Tukey's post hoc test, in order to compare differences between the rat treatment groups. The analysis results were expressed as Mean  $\pm$  SEM. Results with a  $P$  value smaller than 0.05 were considered as significant, when considering the differences between the groups.

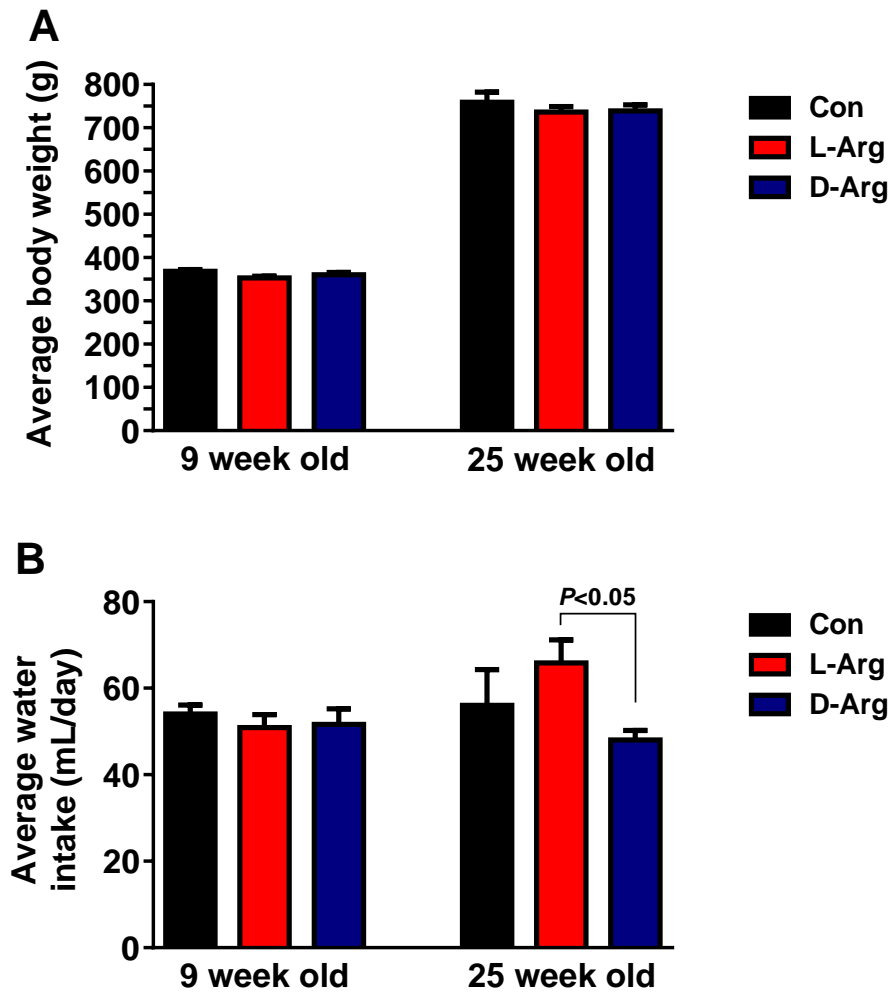
## CHAPTER 3: RESULTS

### 3.1 Oral L- and D-arginine supplements did not significantly affect the average body weight of Sprague-Dawley rats.

The 9-week-old rats were initially randomly divided into three treatment groups and the average body weights of the three groups were not significantly different from each other. Treatment with L- or D-Arg for 16 weeks did not affect the average body weight of the respective treatment group compared with the control group, which received plain drinking water, or compared with each other (Fig. 9A).

### 3.2 Oral D-arginine significantly reduced the average daily water consumption of Sprague-Dawley rats.

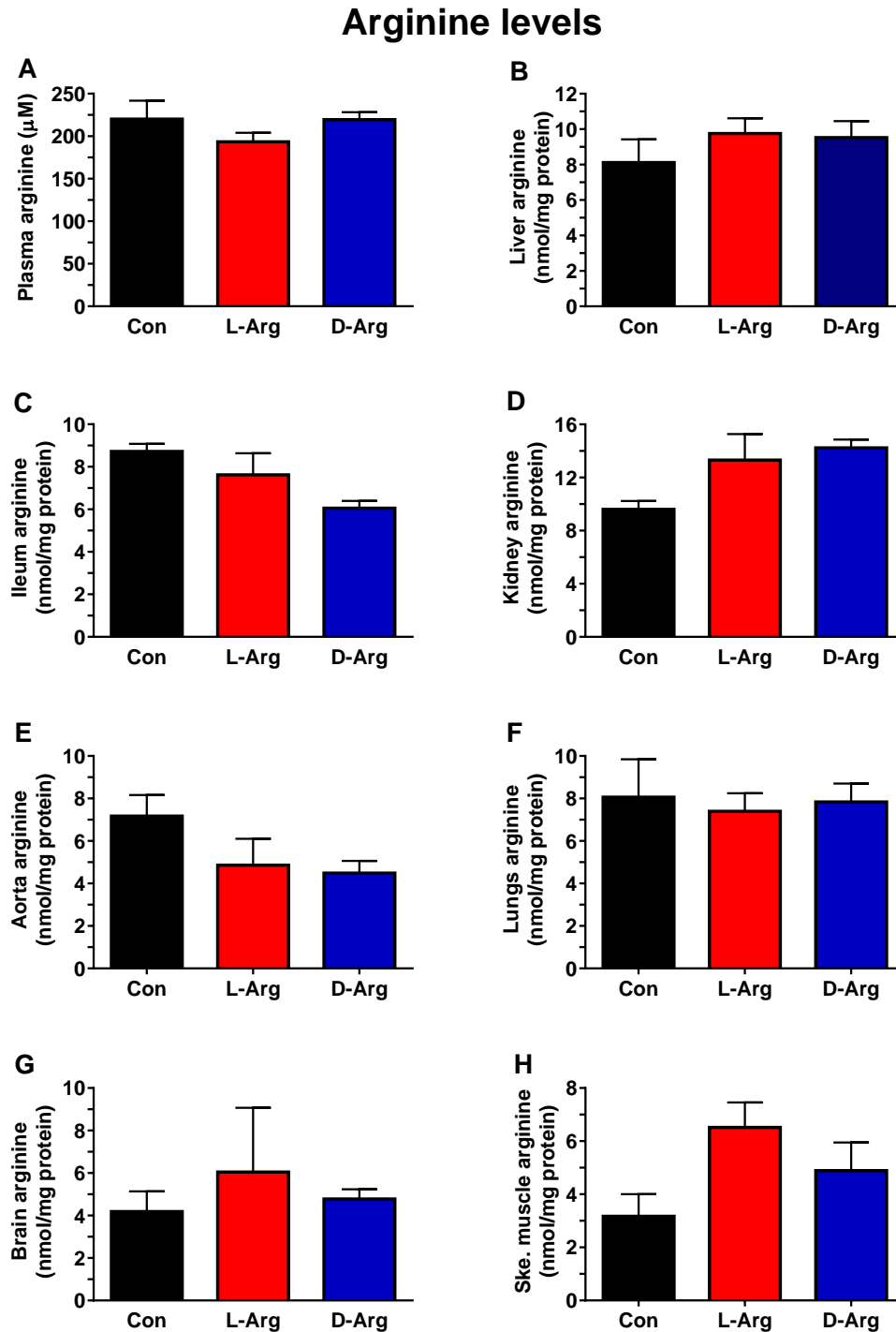
The daily water intake was measured every other day and averaged for the group. The average daily intake was not different among the three treatment groups at the beginning of treatment at 9 weeks of age. Treatment with D-Arg for 16 weeks significantly reduced the average daily water intake, when measured at the end of the treatment period, compared to the L-Arg group (Fig. 9B).



**Figure 9. Oral D-arginine decreased average daily water intake, but arginine did not affect average body weight of Sprague-Dawley rats.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. The average body for each group was calculated from the sum of weights of individual rats in each group divided by the total number. For water intake, the rats were housed individually in separate cages and water intake was recorded every other day. The daily water intake for each rat was calculated and the group average was obtained. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.3 Oral L- and D-arginine did not significantly affect arginine levels in the plasma and different organs.

As shown in Fig. 10, the levels of arginine in the plasma, liver, ileum, kidney, aorta, lungs, brain and skeletal muscle were not significantly affected by 16 weeks of treatment with L-Arg or D-Arg compared with the control group or compared with each other.

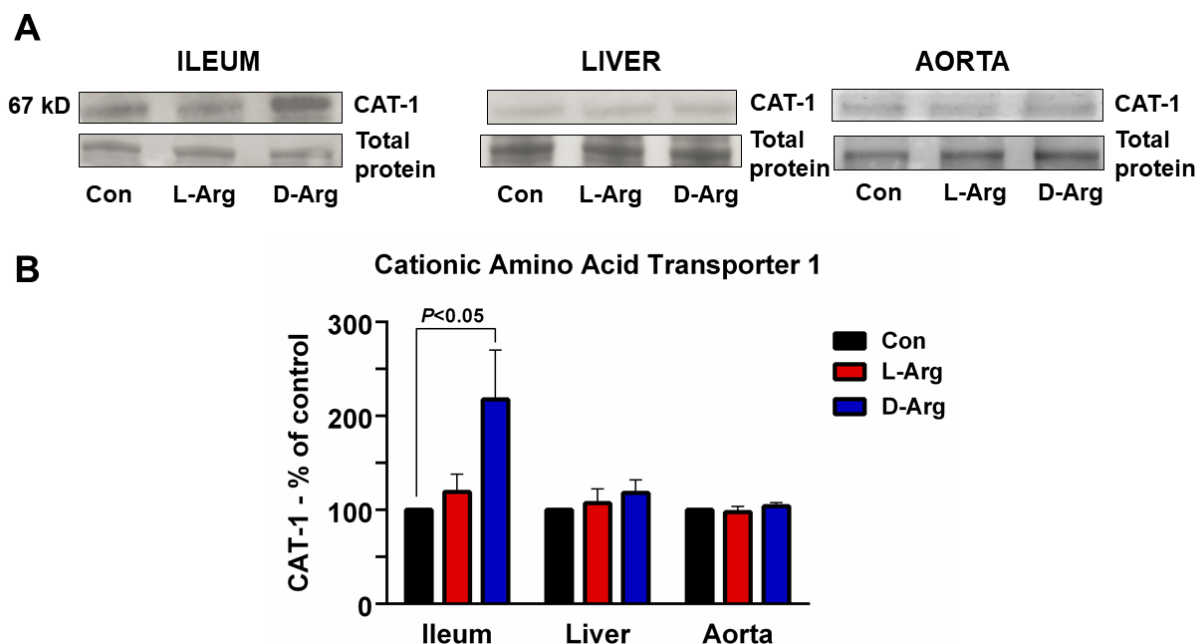


**Figure 10. Oral arginine did not affect the levels of arginine in the plasma and different organs of Sprague-Dawley rats.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Arginine levels were measured

with a specific assay kit (Cat. # ab252892, Abcam Inc., Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups). (Ske. muscle – skeletal muscle).

### 3.4 Oral D-arginine significantly increased cationic amino acid transporter 1 expression in the ileum, but not in the liver or aorta.

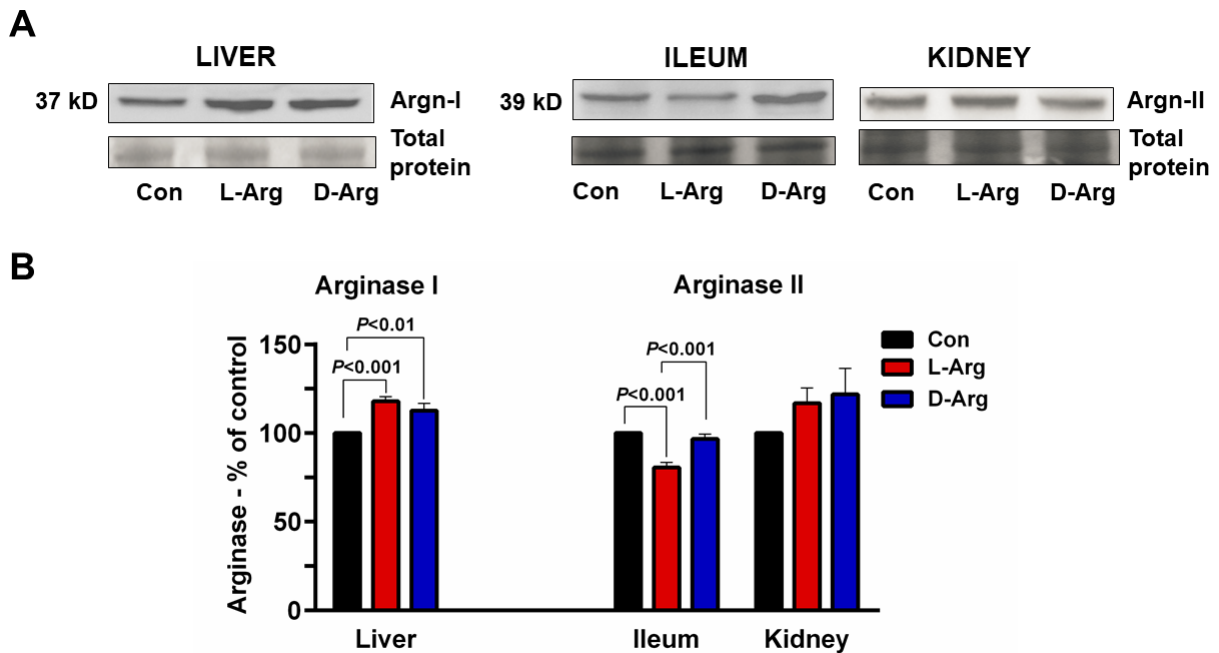
CAT-1 is the main transporter of arginine. In the ileum, the expression of CAT-1 was significantly increased in the D-Arg treatment group compared to the control group (Fig. 11A, B). The expression of CAT-1 in the liver and the aorta was not affected by treatment with L-Arg or D-Arg (Fig. 11A, B).



**Figure 11. Oral D-arginine increased the expression of cationic amino acid transporter 1 in the ileum.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Western blotting was performed using a specific anti-cationic amino acid transporter-1 [CAT-1 (SLC7A1), 1:1000, Cat. # ABIN5965961, Antibodies-Online Inc., Atlanta, GA, USA] antibody as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.5 Oral L- and D-arginine significantly affected arginase expression in the liver and the ileum in Sprague-Dawley rats.

The expression of arginase I was significantly increased in the liver following treatment with both L-Arg and D-Arg, compared with the control group (Fig. 12A, B). On the other hand, treatment with L-Arg for 16 weeks significantly decreased the expression of arginase II in the ileum, compared to the control group and the D-Arg group (Fig. 12A, B). D-Arg did not affect arginase II expression in the ileum (Fig. 12A, B). L-Arg and D-Arg did not affect arginase II expression in the kidney (Fig. 12A, B).



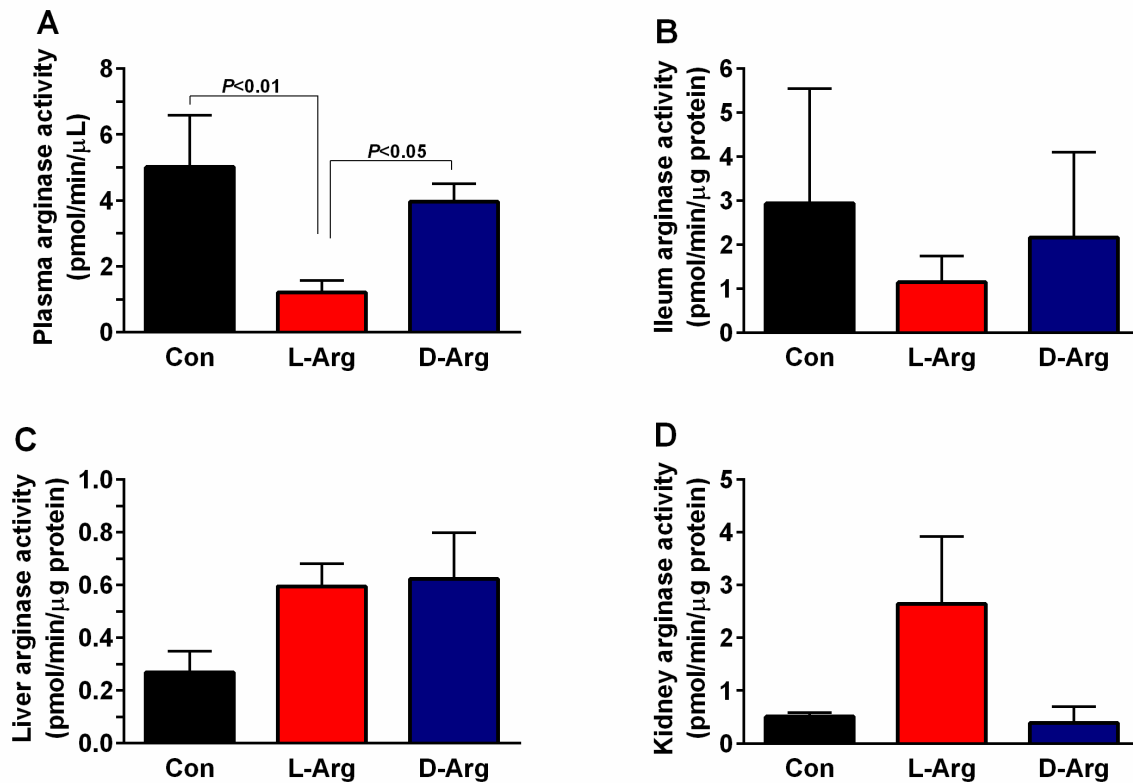
**Figure 12. Oral L- and D-arginine increased arginase expression in the liver, but L-arginine decreased it in the ileum.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Western blotting was performed using specific anti-arginase I and anti-arginase-II antibodies (1:1000, Cat. # ab91279 and Cat. # ab203071, respectively, Abcam Inc. Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.



### 3.6 Oral L-arginine significantly decreased arginase activity in the plasma, but not in the ileum, liver or kidney.

The activity of arginase in the plasma was significantly reduced for the L-Arg group of SD rats treated for 16 weeks, when compared with both the control and D-Arg groups (Figure 13A). However, the activity levels of this enzyme were not significantly altered in the ileum (Fig. 13B), liver (Fig. 13C) or kidney (Fig. 13D).

## Arginase activity

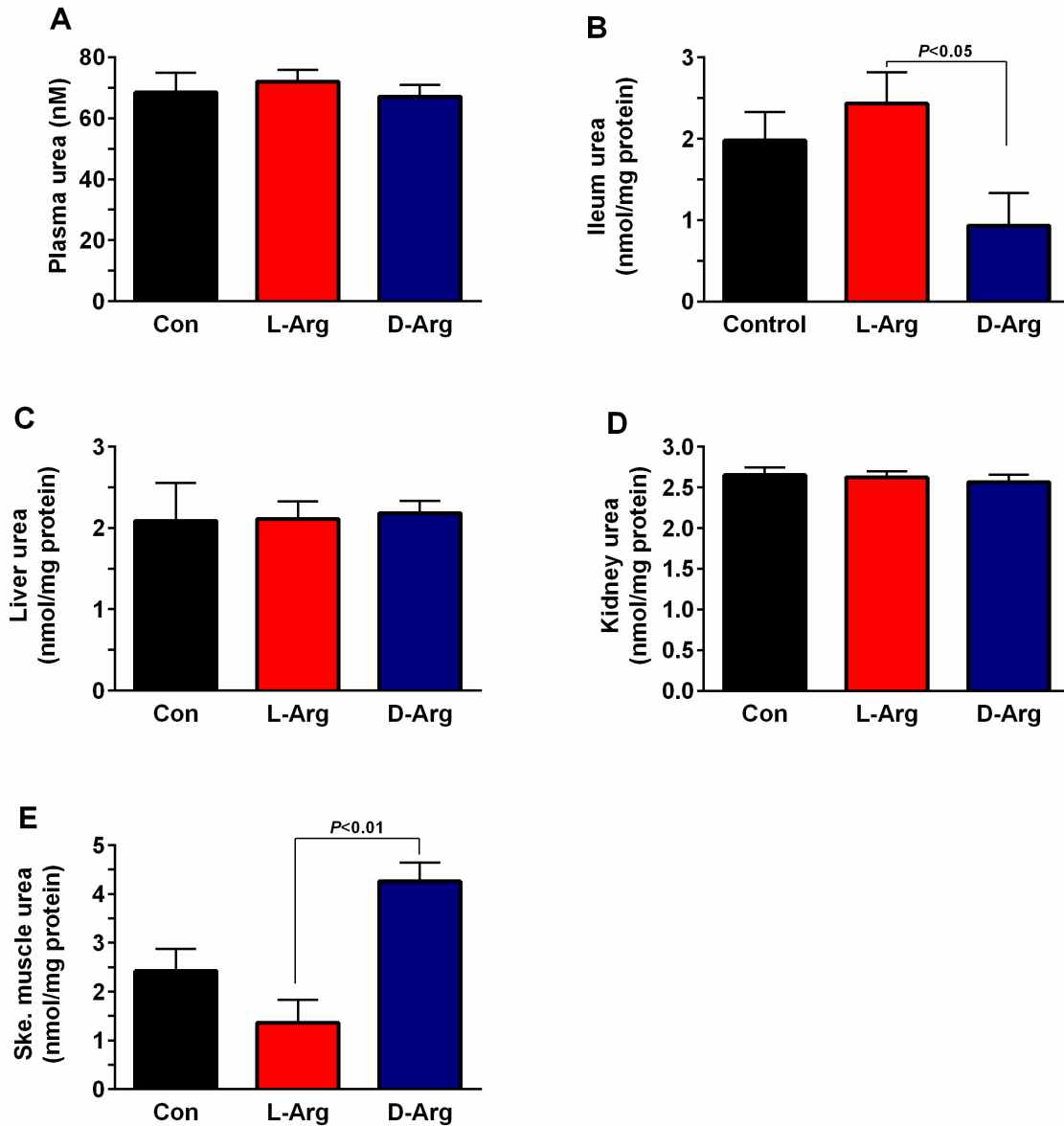


**Figure 13. Oral L-arginine decreased arginase activity in the plasma.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. An arginase activity assay was performed with a specific assay kit (Cat. # ab180877, Abcam Inc., Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.7 Oral D-arginine significantly affected urea levels in the ileum and the skeletal muscle in Sprague-Dawley rats.

Urea is a product of the arginine-arginase metabolic pathway. Treatment with D-Arg for 16 weeks significantly decreased urea levels in the ileum compared with the L-Arg group, but not the control group (Fig. 14B). On the other hand, the D-Arg group had significantly higher urea levels in the skeletal muscle compared with the L-Arg group, but not the control group (Fig. 14E). L-Arg and D-Arg did not affect urea levels in the plasma, liver and kidney (Fig. 14A, C, D).

## Urea levels



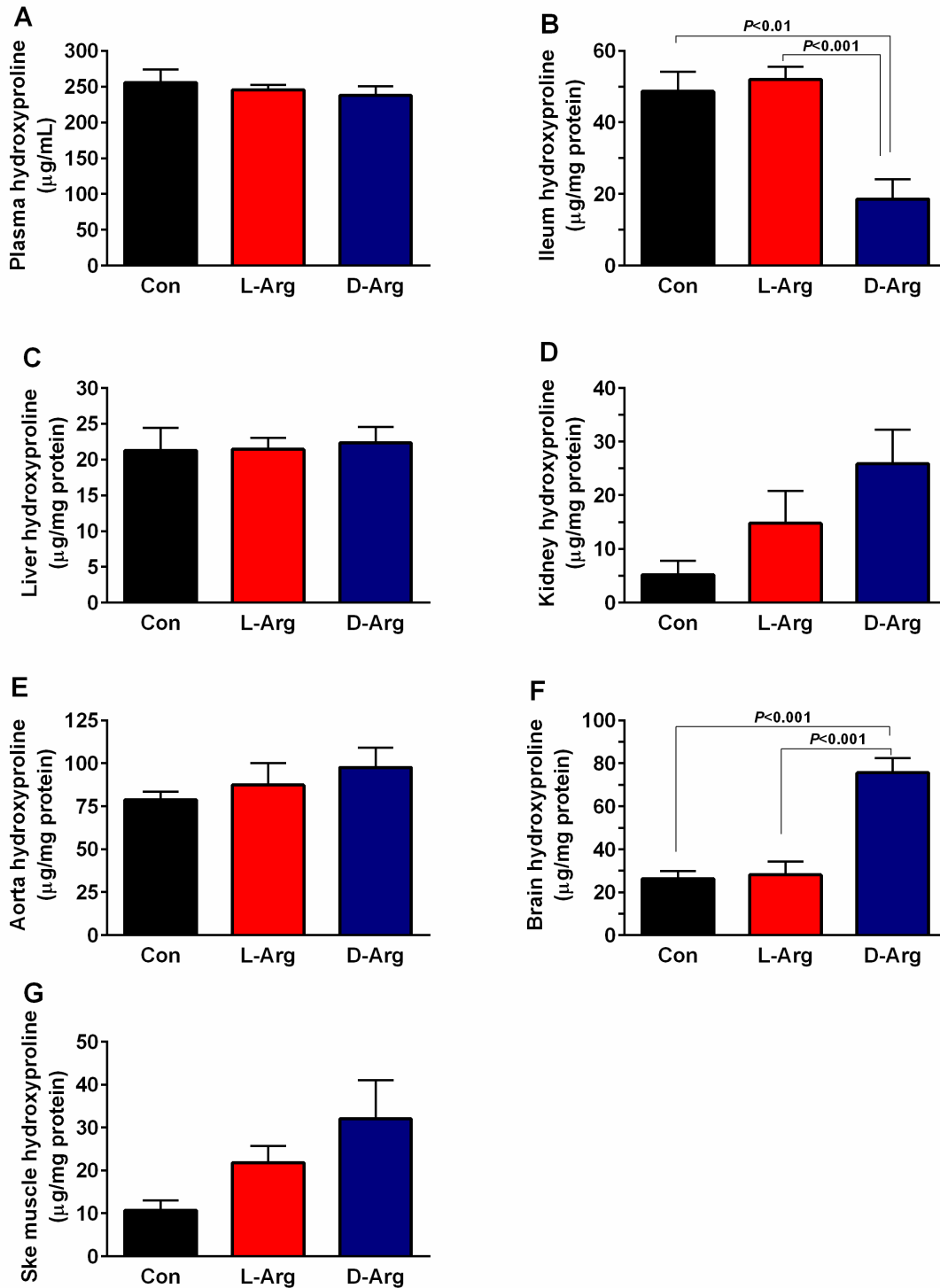
**Figure 14. Oral D-arginine decreased urea levels in the ileum, but increased it in the skeletal muscle, compared to L-arginine.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Urea levels were measured with a specific assay kit (Cat. # ab83362, Abcam Inc., Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.8 Oral D-arginine significantly affected hydroxyproline levels in the ileum and the brain.

Hydroxyproline is a derivative of proline, which is formed from L-Arg catalyzed by arginase and OAT. Treatment with oral D-Arg for 16 weeks significantly decreased hydroxyproline levels in the ileum compared to the control as well as the L-Arg group (Fig. 15B). On the other hand, D-Arg significantly increased hydroxyproline levels in the brain, compared with the control and the L-Arg groups (Fig. 15F).

L-Arg and D-Arg treatment did not affect hydroxyproline levels in the plasma, liver, kidney, aorta or skeletal muscle in comparison to the control group or compared to each other (Fig. 15).

## Hydroxyproline levels

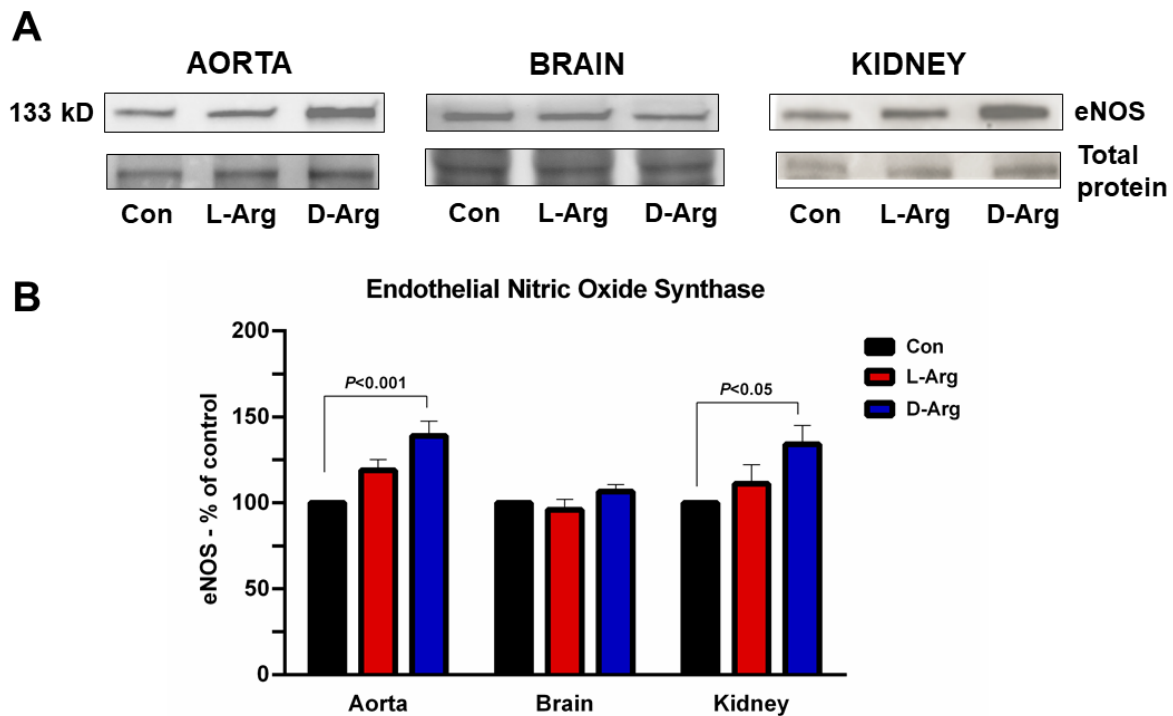


**Figure 15. Oral D-arginine decreased hydroxyproline levels in the ileum, but increased it in the brain.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-

arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Hydroxyproline levels were measured with a specific assay kit (Cat. # ab222941, Abcam Inc., Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.9 Oral D-arginine significantly increased endothelial nitric oxide synthase expression in the aorta and the kidney, but not in the brain.

NOSs are important enzymes catalyzing the formation of NO from L-Arg. In both the aorta and kidney, the expression of eNOS was significantly increased compared to the control group, following oral D-Arg treatment for 16 weeks, but not L-Arg (Fig. 16). Conversely, in the brain, neither L-Arg nor D-Arg significantly affected the expression of eNOS (Fig. 16).



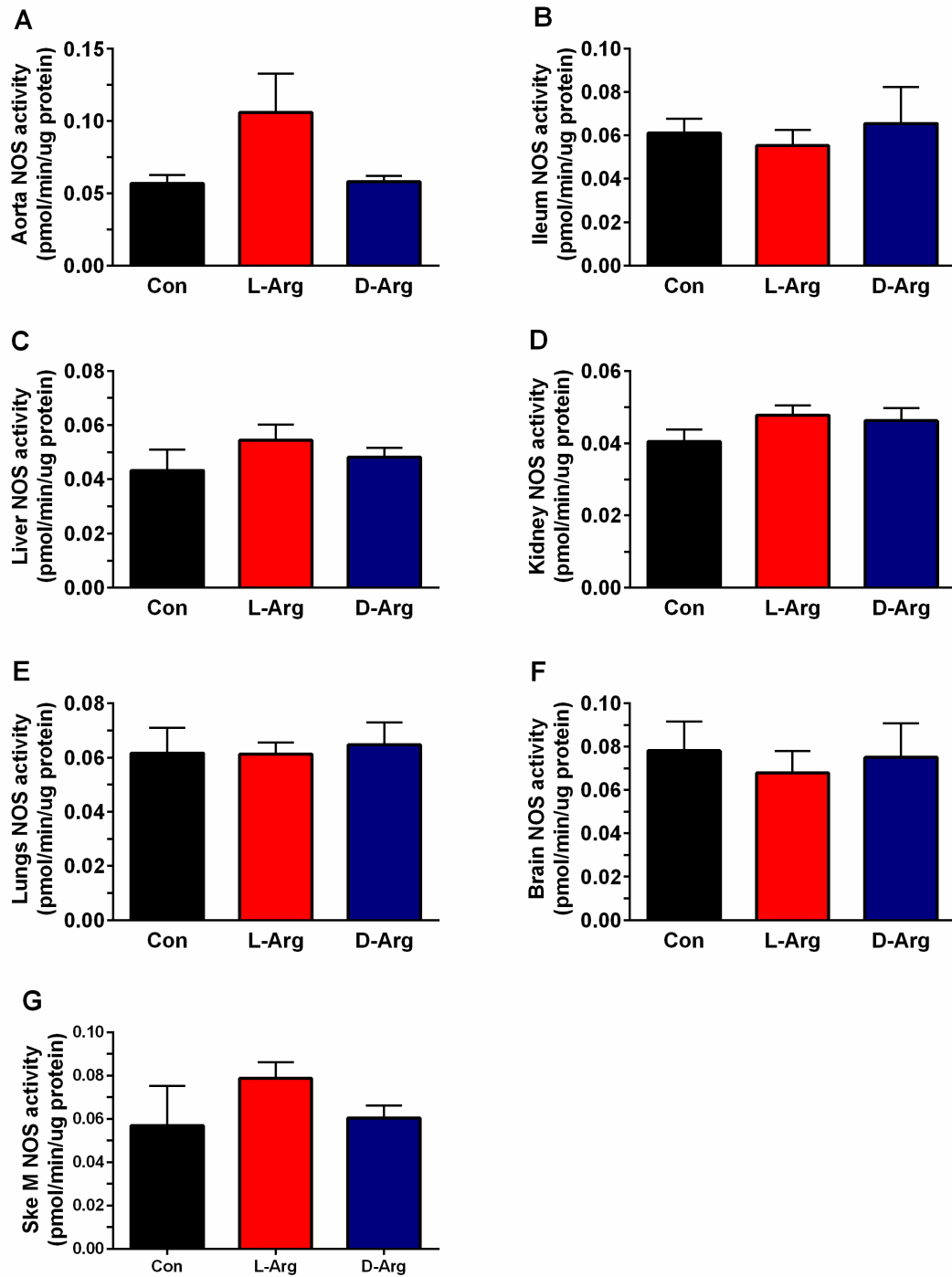
**Figure 16. Oral D-arginine increased endothelial nitric oxide synthase expression in the aorta and the kidney.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Western blotting was performed using specific

anti-endothelial nitric oxide synthase (eNOS) antibody (1:500, Cat. # 611852, BD Transduction Laboratories, Mississauga, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n$  = 4 for Con and  $n$  = 7 each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.10 Oral arginine did not affect nitric oxide synthase activity in different organs in Sprague-Dawley rats.

As shown in the graphs in Fig. 17, oral L-Arg or D-Arg treatment for 16 weeks did not affect NOS activity in the aorta, ileum, liver, kidney, lungs, brain and skeletal muscle compared to the control or compared with each other.

## Nitric oxide synthase activity



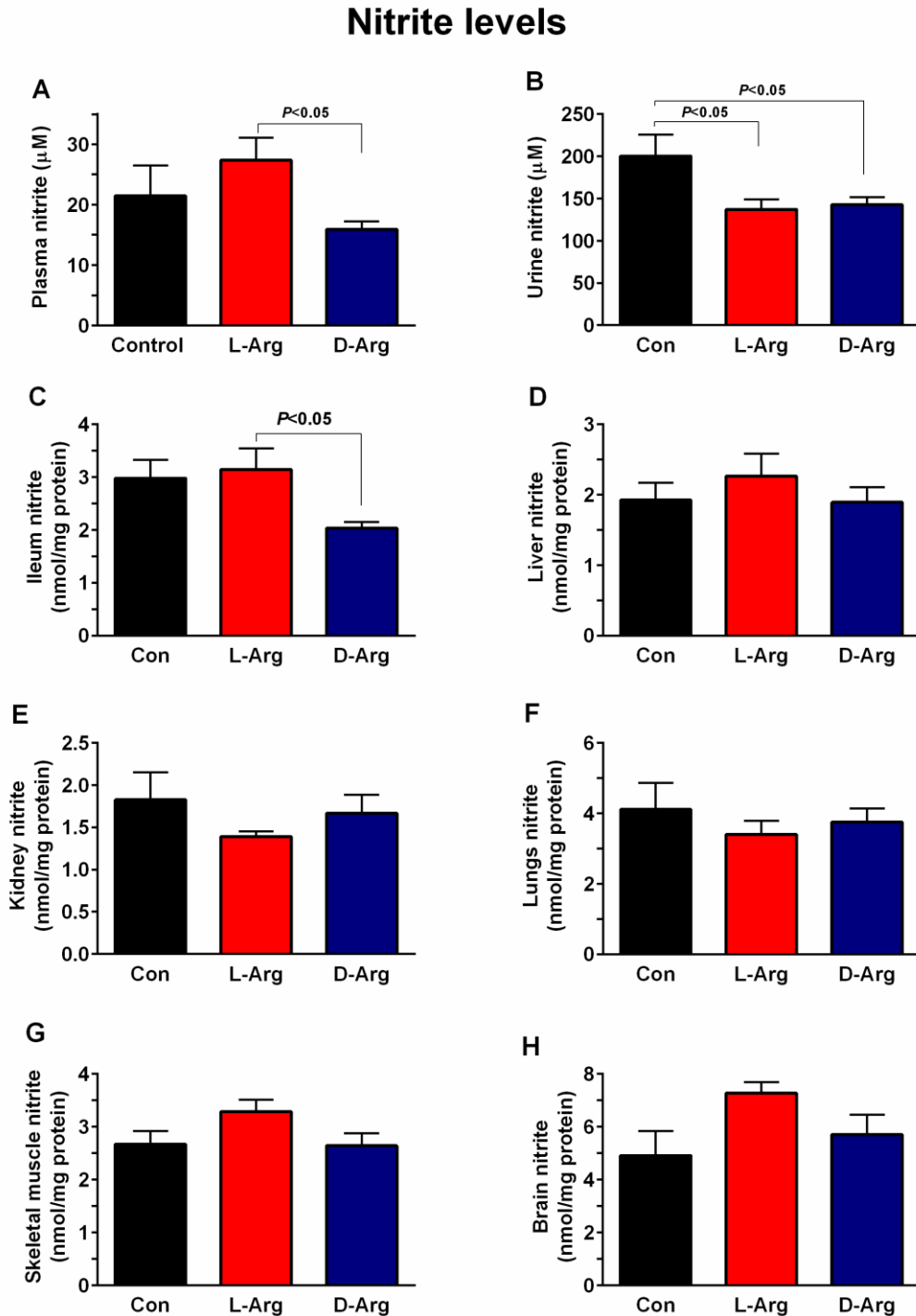
**Figure 17. Oral arginine did not affect the nitric oxide synthase activity in different organs of Sprague-Dawley rats.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine



(L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. A nitric oxide synthase (NOS) activity assay was performed with a specific assay kit (ab211083, Abcam Inc., Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.11 Oral arginine supplements significantly affected nitrate and nitrite levels in the plasma, urine and ileum in Sprague-Dawley rats.

Nitrate and nitrite levels, two main metabolic products of NO, were measured as nitrite, following conversion of nitrate into nitrite in the assay. Nitrite levels were significantly reduced in the plasma (Fig. 18A) and the ileum (Fig. 18C) in the D-Arg treated group, compared with the L-Arg group, but not the control group. Nitrite levels were also significantly decreased in the urine in both the L-Arg and D-Arg groups compared to the control group (Fig. 18B). Nitrite levels in other organs such as the liver, kidney, lungs, skeletal muscle and the brain were not affected by treatment with oral arginine (Fig. 18).

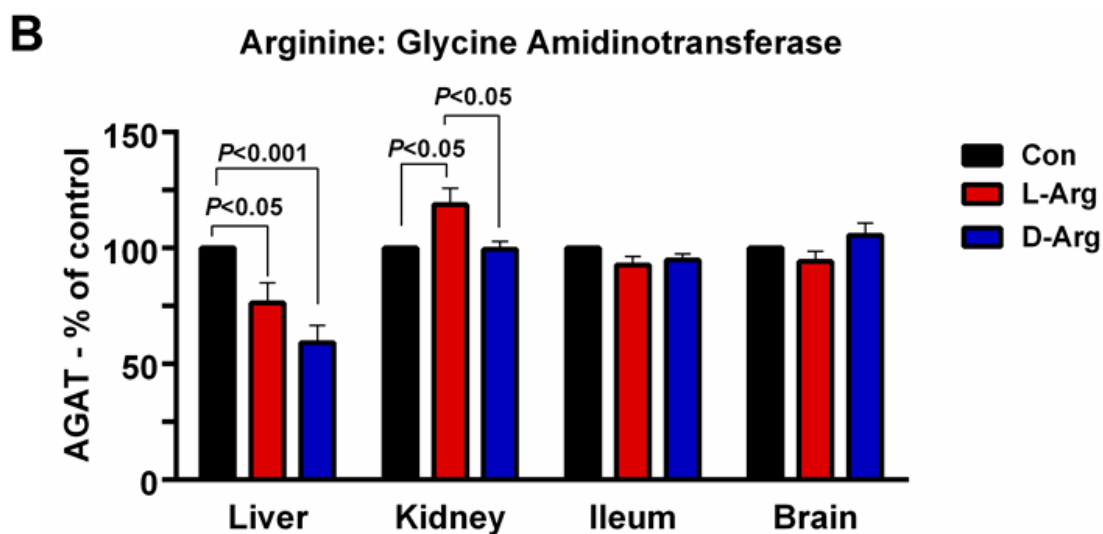
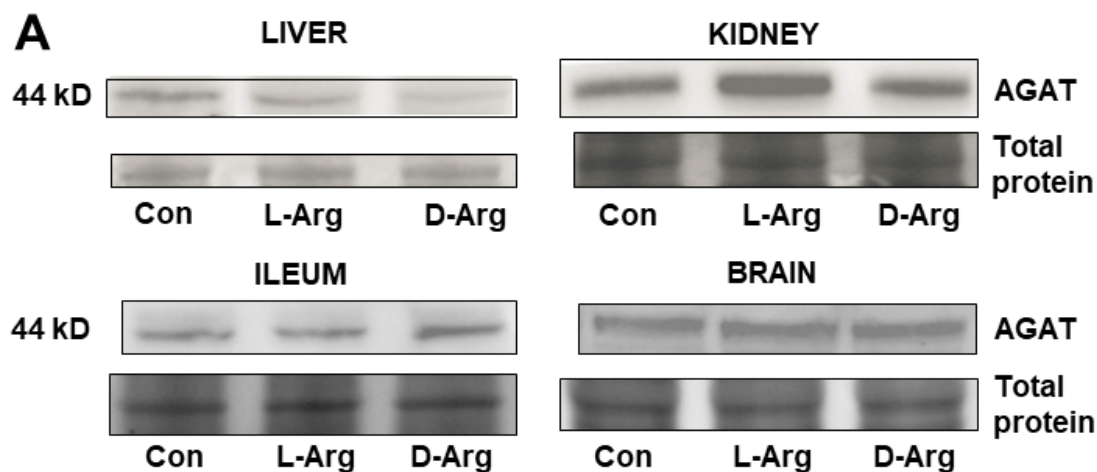


**Figure 18. Oral arginine decreased nitrite levels in the urine.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Nitrate plus nitrite levels were measured as nitrite after conversion of nitrate to nitrite with

nitrate reductase and measured with a specific assay kit (Cat. # 780001, Cayman Chemical, Ann Arbor, MI, USA) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.12 Oral arginine supplements affected the expression of arginine:glycine amidinotransferase in the liver and the kidney.

The expression of AGAT was significantly decreased in the liver following treatment with both, L-Arg and D-Arg, compared to the control group (Fig. 19A, B). However, in the kidney oral L-Arg significantly increased the expression of AGAT compared to the control as well as the D-Arg groups (Fig. 19A, B). On the other hand, the expression of AGAT was not affected in the ileum and the brain by oral arginine (Fig. 19).

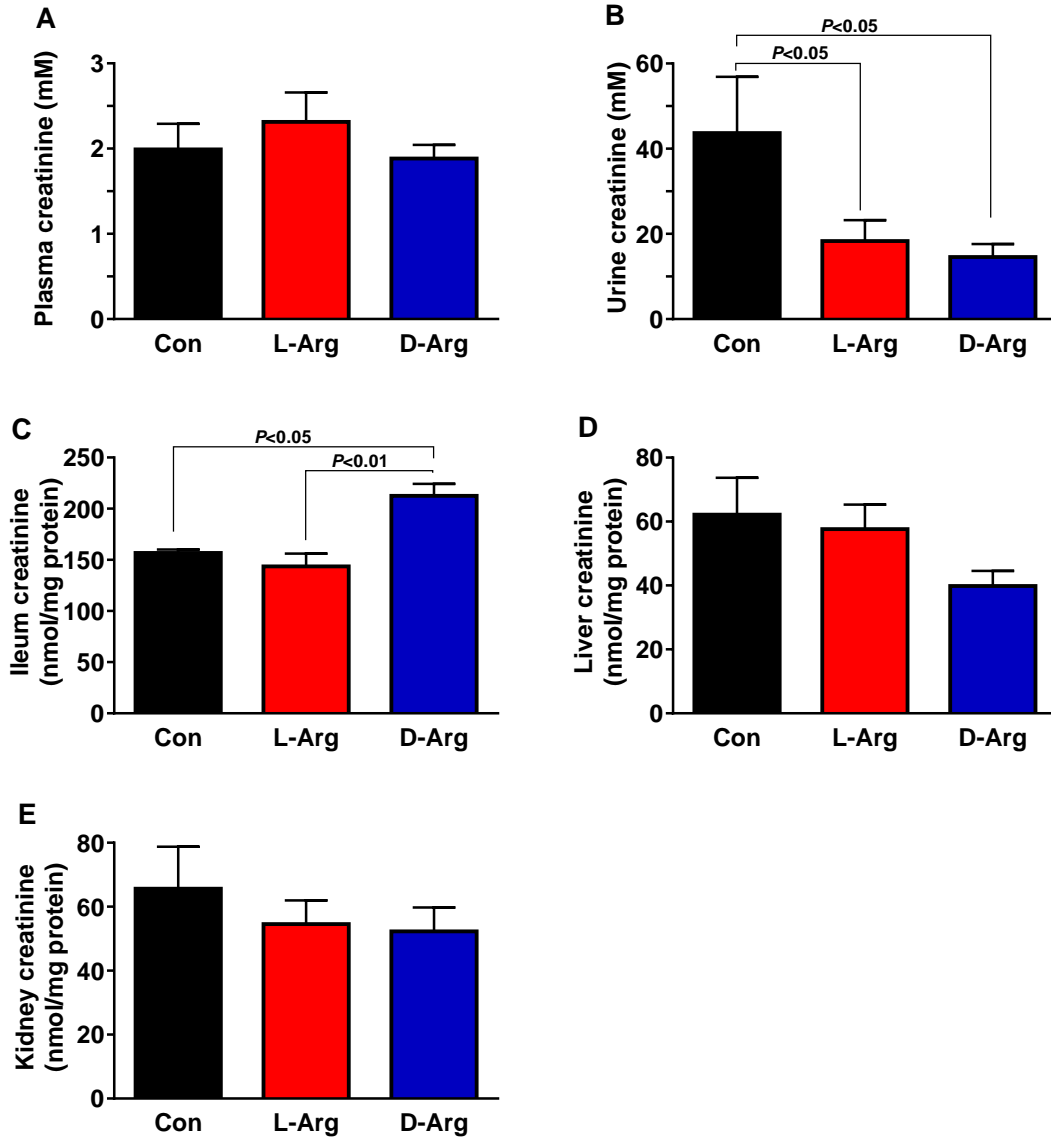


**Figure 19. Oral L- and D-arginine decreased arginine:glycine amidinotransferase expression in the liver, but L-arginine increased it in the kidney.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Western blotting was performed using specific anti-arginine:glycine amidinotransferase (AGAT) antibody (1:1000, Cat. # ab87062, Abcam Inc., Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.13 Oral arginine significantly affected creatinine levels in the urine and the ileum.

Creatinine is a product of creatine, which is formed from L-Arg catalyzed by AGAT. The levels of creatinine were significantly decreased in the urine of L-Arg and D-Arg treated groups, compared to the control group (Fig. 20B). On the other hand, D-Arg treatment significantly increased creatinine levels in the ileum compared to the control and the L-Arg groups (Fig. 20C). Creatinine levels in the plasma, liver and kidney were not affected by L-Arg or D-Arg treatment (Fig. 20).

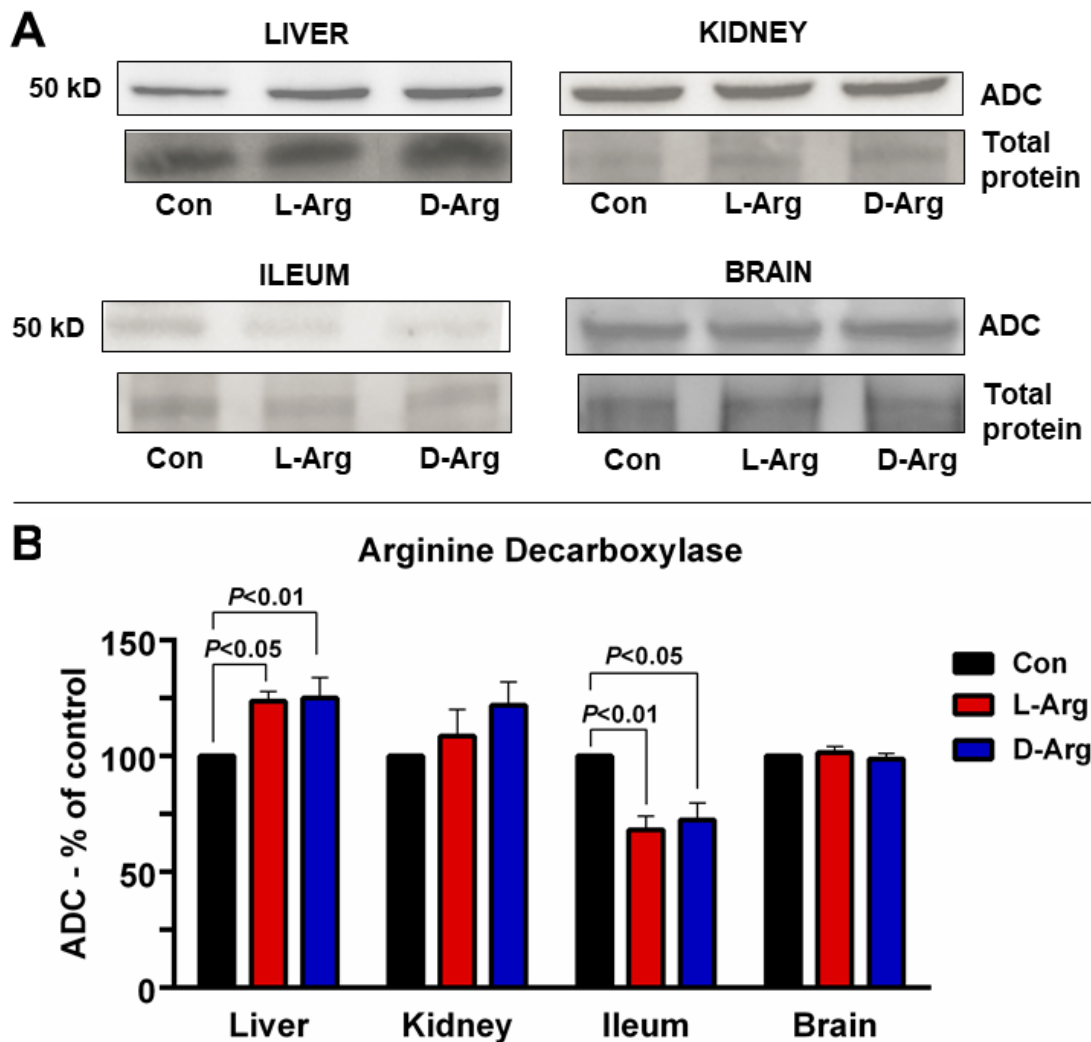
## Creatinine levels



**Figure 20. Oral L- and D-arginine decreased creatinine levels in the urine, but D-arginine increased it in the ileum.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Creatinine levels were measured with a specific assay kit (Cat. # ab65340, Abcam Inc., Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.14 Oral arginine significantly affected the expression of arginine decarboxylase in the liver and the ileum.

The expression of ADC was significantly increased in the liver following treatment with oral L-Arg and D-Arg, compared with the control group (Fig. 21A, B). However, both L-Arg and D-Arg significantly decreased the expression of ADC in the ileum, compared to the control group (Fig. 21A, B). The expression of ADC in the kidney and the brain was not affected by L-Arg or D-Arg (Fig. 21).



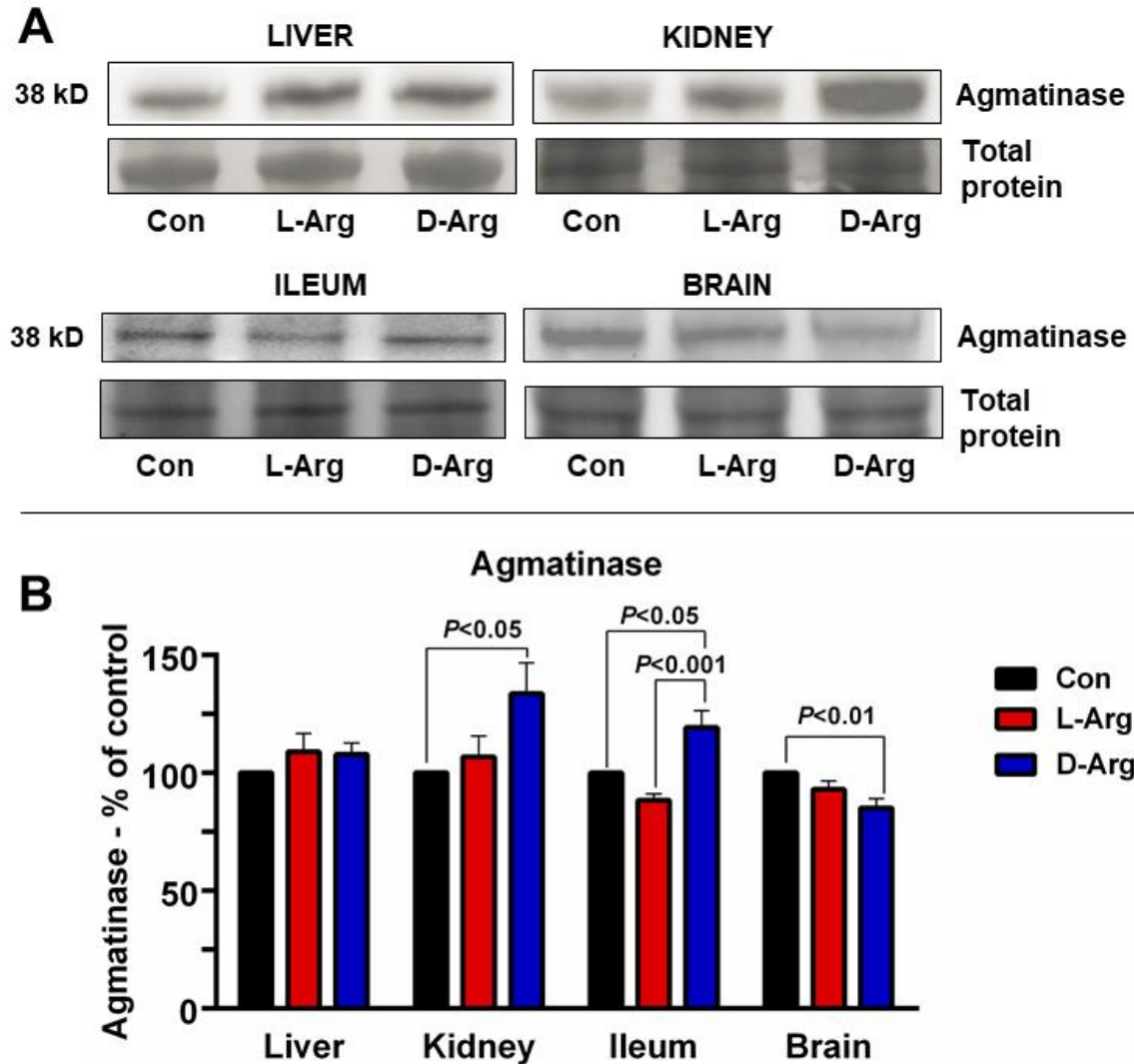
**Figure 21. Oral arginine increased arginine decarboxylase expression in the liver, but decreased it in the ileum.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Western blotting was performed using

specific anti-arginine decarboxylase (ADC) antibody (1:1000, Cat. # ab157214, Abcam Inc., Toronto, ON, Canada), as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.15 Oral arginine significantly affected the expression of agmatinase in the kidney, ileum and brain in rats.

Treatment with oral D-Arg, but not L-Arg, significantly increased the expression of agmatinase in the kidney, compared to the control group (Fig. 22A, B). In the ileum, D-Arg increased agmatinase expression compared to the control as well as the L-Arg group (Fig. 22A, B). In the brain, D-Arg had the opposite effect of decreasing agmatinase expression, compared to the control group (Fig. 22A, B). Agmatinase expression in the liver was not affected by oral L-Arg or D-Arg treatment (Fig. 22).



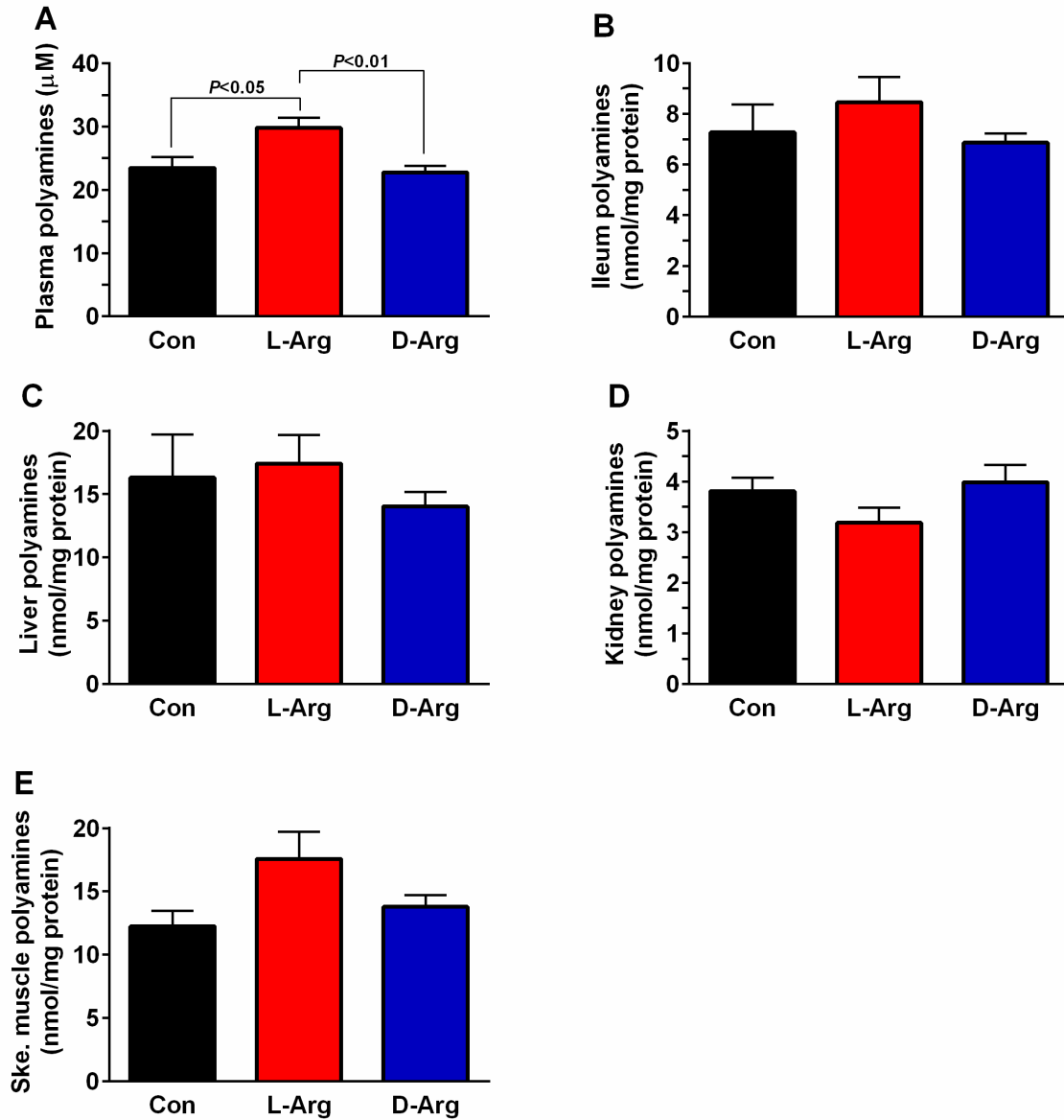


**Figure 22. Oral D-arginine increased agmatinase expression in the kidney and the ileum, but decreased it in the brain.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Western blotting was performed using specific anti-agmatinase antibody (1:1000, Cat. # ab231894, Abcam Inc., Toronto, ON, Canada), as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.16 Oral L-arginine significantly increased total polyamines levels in the plasma.

Putrescine, spermine and spermidine are polyamine products of L-Arg metabolism catalyzed by ADC and agmatinase. The levels of total polyamines were significantly elevated in the plasma for the L-Arg treated group of SD rats, compared to the control and the D-Arg groups (Fig. 23A). Total polyamine levels in the ileum, liver, kidney and skeletal muscle were not affected by treatment with oral L-Arg and D-Arg (Fig. 23).

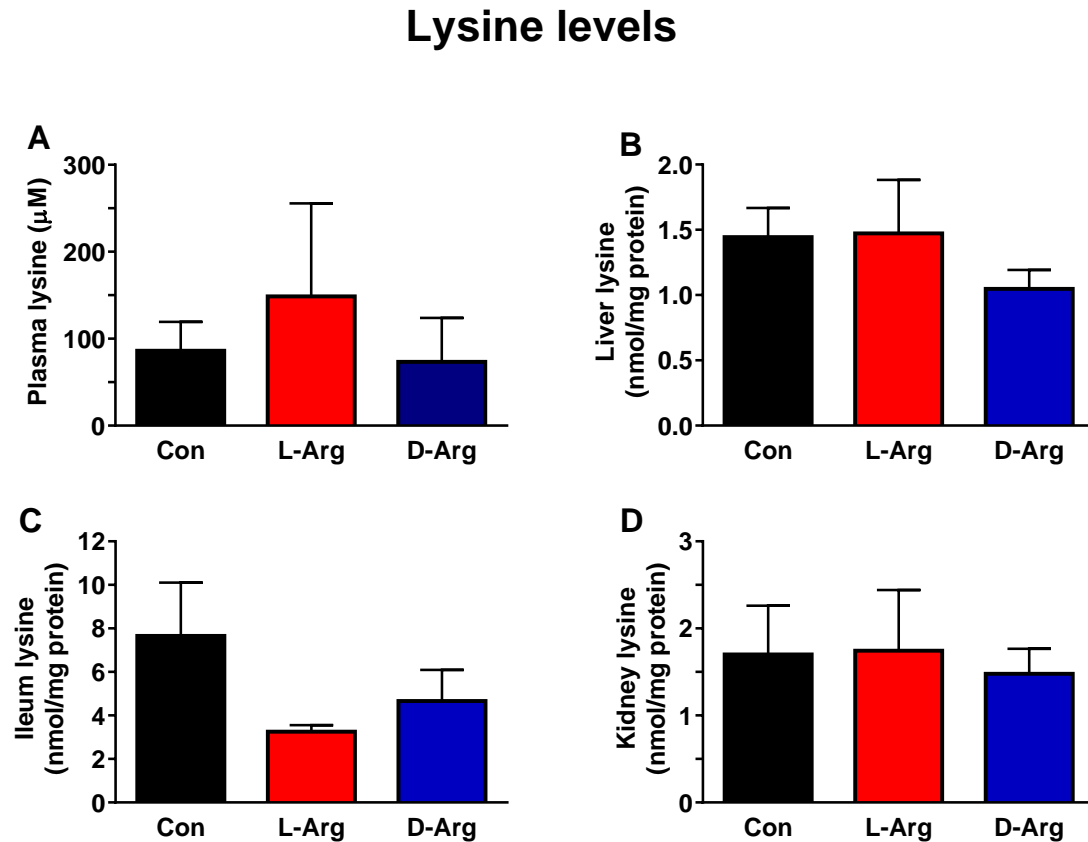
## Total polyamines



**Figure 23. Oral L-arginine increased total polyamine levels in the plasma.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Total polyamines levels were measured with a specific assay kit (Cat. # ab239728, Abcam Inc., Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.17 Oral arginine did not affect lysine levels in the plasma and organs in rats.

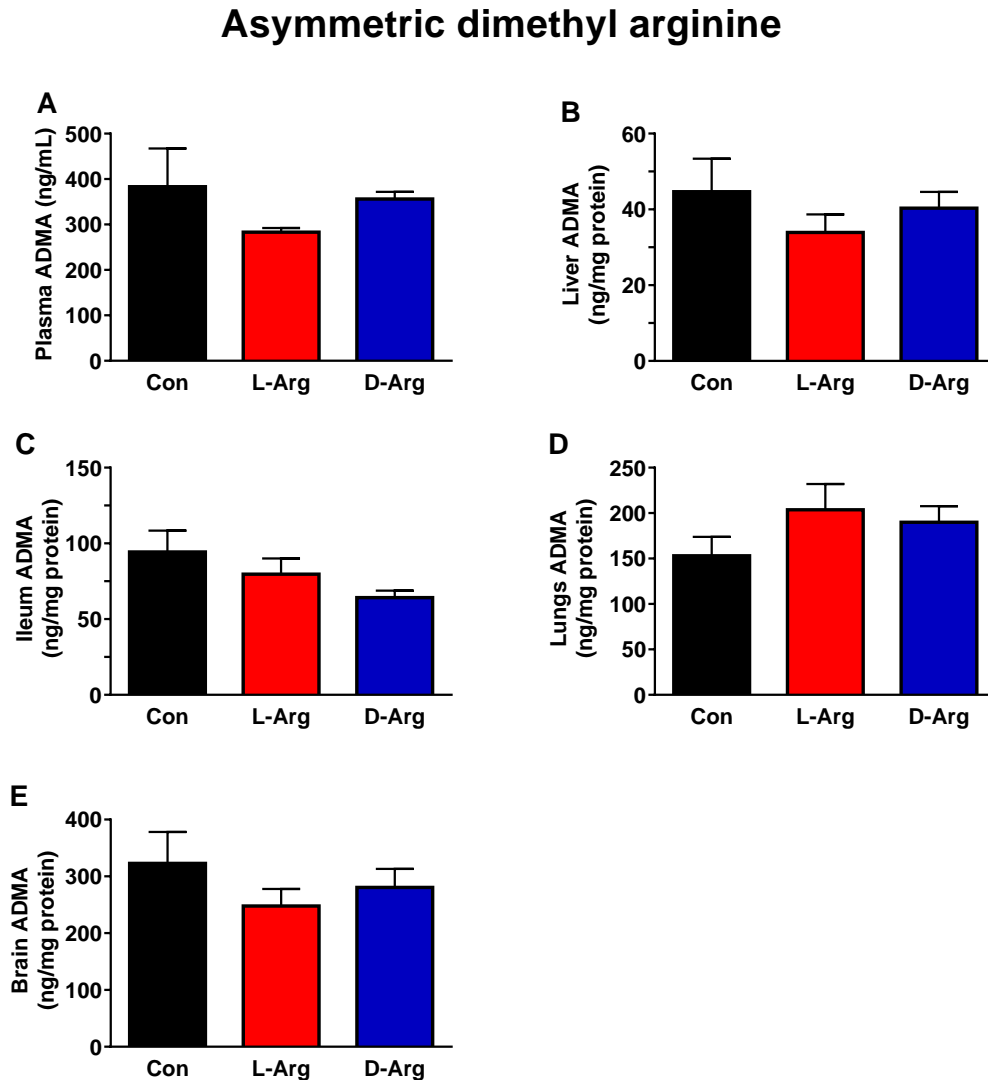
Lysine is transported by the same CAT-1 transporter as arginine, leading to competition. Oral L-Arg or D-Arg treatment for 16 weeks did not affect lysine levels in the plasma, liver, ileum or kidney (Fig. 24).



**Figure 24. Oral arginine did not affect lysine levels in the plasma or different organs of Sprague-Dawley rats.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. Lysine levels were measured with a specific assay kit (Cat. # ab273311, Abcam Inc., Toronto, ON, Canada) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

### 3.18 Oral arginine did not affect asymmetric dimethylarginine levels in the plasma and organs in rats.

ADMA is formed by methylation of arginine during protein modification and could compete with L-Arg for NOS. As shown in the graphs in Fig. 25, treatment with oral L-Arg or D-Arg for 16 weeks did not affect the levels of ADMA in the plasma, liver, ileum, lungs and brain in rats, when compared with to the control group, as well as each other.



**Figure 25. Oral arginine did not affect the levels of asymmetric dimethylarginine in the plasma and different organs of Sprague-Dawley rats.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each

in drinking water for 16 weeks. The control (Con) group received plain drinking water. Asymmetric dimethylarginine (ADMA) levels were measured with a specific assay kit (Cat. # OKEH02587, Aviva Systems Biology, San Diego, CA, USA) as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups).  $P < 0.05$  was considered significant.

**Table 2. Physiological effects of oral L-Arg and D-Arg on its metabolic pathways: Summary of results obtained with oral arginine at 1000 mg/kg/day for 16 weeks in Sprague-Dawley rats, in comparison with oral arginine at 500 mg/kg/day for 4 weeks study (Martin & Desai, 2020)**

Parameter	Oral L-Arg	Oral D-Arg
Body Weight	↔	↔
Water Intake	↔	↓ vs. L-Arg (↔)
Arginine Levels	↔	↔
Lysine Levels	↔	↔
ADMA Levels	↔	↔
CAT-1 Expression	↔ (↓ Li, Il)	↑ Il (↔)
<b>Arginase Pathway</b>		
Arginase Expression	↑ Li ↓ Il vs. C, D-Arg (↓ Il)	↑ Li ↑ Il vs. L-Arg (↓ Li, Il)
Arginase Activity	↓ Pl vs. C, D-Arg (↔)	↑ Pl vs. L-Arg (↔)
Urea Levels	↑ Il vs. D-Arg ↓ Sk.M vs. D-Arg (↔)	↓ Il vs. L-Arg ↑ Sk.M vs. L-Arg (↑ Li; ↑ Ki vs. C, L-Arg)
Hydroxyproline Levels	↑ Il vs. D-Arg ↓ Br vs. D-Arg	↓ Il vs. C, L-Arg ↑ Br vs. C, L-Arg
<b>eNOS Pathway</b>		
eNOS Expression	↔ (↑ Ao, Ki)	↑ Ao, Ki (↑ Ki)
NOS Activity	↔	↔

	(↓ Lu vs. D-Arg)	(↑ Lu vs. C, L-Arg)
<b>Nitrate + Nitrite Levels</b>	↑ Pl, Il vs. D-Arg ↓ Ur (↑ Pl vs C, D-Arg)	↓ Pl, Il vs. L-Arg ↓ Ur (↓ Pl vs. L-Arg)
<b>ADC Pathway</b>		
<b>ADC Expression</b>	↑ Li, ↓ Il (↑ Li)	↑ Li, ↓ Il (↔)
<b>Agmatinase Expression</b>	↓ Il vs. D-Arg (↔)	↑ Ki, ↓ Br ↑ Il vs. C, L-Arg (↑ Li vs L-Arg)
<b>Total Polyamines Levels</b>	↑ Pl vs. C, D-Arg (↑ Li vs C, D-Arg)	↓ Pl vs. L-Arg (↑ Pl)
<b>AGAT Pathway</b>		
<b>AGAT Expression</b>	↓ Li ↑ Ki vs. C, D-Arg (↓ Li, Ki)	↓ Li ↓ Ki vs. L-Arg (↑ Li vs L-Arg ↑ Ki vs C, L-Arg)
<b>Creatinine Levels</b>	↓ Ur ↓ Il vs. D-Arg (↓ Sk)	↓ Ur ↑ Il vs. C, L-Arg (↑ Li)

Results shown in black font are from the current study obtained with a dose of oral L-Arg or D-Arg at 1000 mg/kg/day administered for 16 weeks to Sprague-Dawley rats. Results from the lower dose arginine study (500 mg/kg/day for 4 weeks) published from the supervisor's lab (Martin & Desai, 2020) have been included in the table of summary, highlighted in red in bracket, to compare with the results of the current study.

**Symbols:** ↔ no change compared to control; ↑ increase compared to control unless otherwise stated; ↓ decrease compared to control unless otherwise stated

**Abbreviations:** ADC – arginine decarboxylase; ADMA – asymmetric dimethylarginine; AGAT – arginine: glycine amidinotransferase; Ao – aorta; Br – brain; C – control; CAT-1 – cationic



amino acid transporter 1; D-Arg – D-arginine; eNOS – endothelial nitric oxide synthase; Il – ileum;  
Ki – kidney; L-Arg – L-arginine; Li – liver; Pl – plasma; Sk. M – skeletal muscle

## CHAPTER 4: DISCUSSION

Oral arginine supplements are widely being used by healthy people such as athletes, and people with different disease conditions such as hypertension, erectile dysfunction and MELAS, as a few examples. The use of oral arginine is based on its known physiological functions and the widely published reports as a substrate of NOSs and a precursor for NO formation and vasodilation. Media reports of these effects and especially the news that NO mediates penile erection, resulted in unregulated use of NO enhancing strategies to boost NO availability, including widespread use of arginine supplements (Klotz et al., 1999). The unfortunate outcome is that there is a general lack of awareness of multiple metabolic pathways for L-Arg and how they would be affected by oral supplements. There still is a huge gap of evidence and knowledge on safety and efficacy of oral arginine supplements, and the recommended doses for use in particular conditions need be further investigated. As well, studies involving oral arginine supplementation often leave out D-Arg from consideration, as D-Arg is commonly known to be physiologically inert in the body. In order to verify this, we tested for the effects of both L- and D-Arg supplementation on the major metabolic pathways of SD rats. I report the physiological effects of chronic supplementation of oral L- and D-Arg in SD rats, treated for 16 weeks at a dose of 1000 mg/kg/day.

Treatment with oral L-Arg or D-Arg at 1000 mg/kg/day for 16 weeks in male SD rats did not produce any overt toxicity. The rats were observed every other day in the vivarium, and they showed normal activity and feeding behaviour among the three groups. There was no mortality.

Firstly, the body weight of the SD rats administered oral L- or D-Arg at 1000 mg/kg/day for 16 weeks was not significantly altered in comparison to the control group, as shown in Fig. 9A. The rats had been randomly divided into three groups and the average weight was not significantly different between groups at the start of treatment at 9 weeks of age (Fig. 9A). Though the weight of the rats from all groups increased with age, there was no significant difference in body weight among the three rat groups at the end of the 16-week treatment period at 25 weeks of age. This result is consistent with the findings from the previously published study from our lab, which reported that neither L- nor D-Arg oral supplementation at 500 mg/kg/day for 4 weeks led to significant changes in body weight of SD rats, in comparison to the control group (Martin and Desai, 2020). It may be safe to assume that oral arginine supplements are apparently not affecting appetite or food intake.

The water intake of rats was measured every other day and to enable this, the rats were housed individually in separate cages. The main purpose to record the water intake was to help us to add the appropriate amount of L-Arg or D-Arg to the drinking water so that each rat consumed the same daily dose of 1000 mg/kg/day of arginine. Thus, rats drinking less water had more arginine administered in their water. Water with arginine was changed every other day to account for any changes in daily drinking water using a specially devised formula described in the methods. As shown in Fig. 9B, the daily average water intake for the three groups was similar at the start of the treatment at 9 weeks of age. However, at the end of the 16-week treatment period when the SD rats were 25 weeks old, the D-Arg group showed significantly lower average daily water consumption in comparison to the L-Arg group, and not the control group (Fig. 9B). One possible reason for this may be due to the distinct smell and yellow colour of the water in which D-Arg was dissolved, unlike the L-Arg powder, which could have possibly deterred the SD rats from drinking water normally, resulting in reduced daily water intake. For both L-Arg and D-Arg, the base was used to make a stock solution of 10 g/L and the pH of the highly alkaline solution was adjusted to 7.4 with hydrochloric acid. In comparison, a dose of 500 mg/kg/day of L-Arg or D-Arg for 4 weeks, reported from this lab, did not affect the average daily water intake of rats in the three groups (Martin and Desai, 2020).

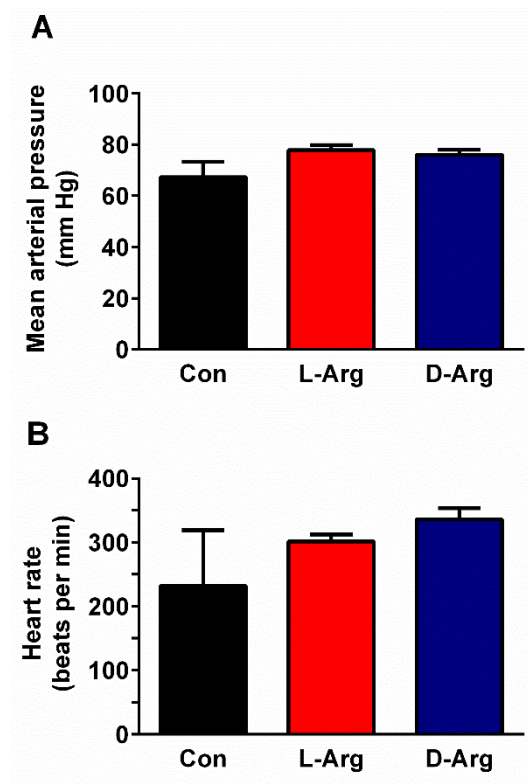
It is important to measure levels of arginine in the body after oral supplementation to determine its absorption and enable correlation of any changes observed with the levels of arginine. Oral L- and D-Arg supplementation at 1000 mg/kg/day for 16 weeks did not significantly affect the normal plasma and organ levels of arginine (Fig. 10). This result can be partly explained by the fact that approximately 40% of the oral arginine in the body gets extensively metabolized in the gut prior to systemic absorption (Castillo et al., 1993b). Additionally, even after absorption, oral arginine in the blood rapidly gets catabolized and cleared from the body (Wu et al., 2007). The elimination half-life of oral arginine supplements has been found to be around 1 hour (Bode-Böger et al., 1998) and so it makes sense that the levels in the plasma remained unchanged after long-term arginine supplementation. Another contributing factor could be changes in the expression of the main transporter for arginine, CAT-1, as described below and in the previous study from our lab (Martin and Desai, 2020). A dose of 500 mg/kg/day for 4 weeks in male SD rats also did not affect arginine levels in the plasma and different organs (Martin and Desai, 2020). This is very significant because it indicates the rapid and dynamic metabolism of arginine and the possibility that the body might

be capable of rapidly adjusting to dietary fluctuations or more stable changes in arginine intake as supplements.

CAT-1 is a primary transporter for arginine (White, 1985). Surprisingly, oral D-Arg, and not L-Arg, significantly increased CAT-1 expression in the ileum in comparison to control (Fig. 11). The reason why ileum was chosen as a site to measure CAT-1 expression in the treated SD rats was because CAT-1 in the small intestine acts as the primary transporter of arginine for its absorption into the systemic circulation (Devés and Boyd, 1998). CAT-1 expression was also measured in the liver and aorta, because the liver is the secondary site containing CAT-1 for further arginine absorption after the initial intestinal uptake, and in the aortic endothelial cells, CAT-1 has been found to colocalize with eNOS (Shin et al., 2011). However, there was no significant effect of arginine supplementation on CAT-1 expression in the liver or aorta (Fig. 11). The finding of upregulation of CAT-1 expression in the ileum with D-Arg but not L-Arg supplementation was unexpected as L-Arg is the isoform known to normally cause metabolic effects in the body, whereas the D-isomer appears to be inert.

On top of that, findings from other studies including the one published with a 500 mg/kg/day dose of arginine study from our lab had shown an opposite effect, that CAT-1 expression was downregulated in response to oral arginine supplementation, possibly in response to the elevated oral arginine levels, similar to a ligand-receptor relationship (Fernandez et al., 2003; Martin and Desai, 2020). The result is also more perplexing with the findings that the levels of neither arginine (Fig. 10) nor lysine (Fig. 24), which are the substrates for this transporter, were altered with L- or D-Arg supplementation. Currently, to the best of our knowledge, there are no research studies that have reported the effects of D-Arg on arginine transporters, and only studies that have reported arginine effects on the upregulation of CAT-1 expression focus specifically on L-Arg (Schwartz et al., 2006; Tachikawa et al., 2018). One possibility is that D-Arg may bind to CAT-1 in the ileum and prevent its interaction with L-Arg, acting as an antagonist, leading to the upregulation of CAT-1 expression. There could be other mechanisms of D-Arg that have not been researched yet. Understanding the pharmacodynamics of D-Arg would definitely be helpful in interpreting its effects on not just the uptake transporters, but also various metabolic enzymes in the body. D-Arg is most definitely an interesting isomer.

Our lab examined the effects of arginine supplements on the mean arterial pressure (MAP). Since, these *in vivo* studies were performed by another student before me, they have not been included in the results section. However, they are interesting to include in this discussion. L-Arg and D-Arg at a dose of 1000 mg/kg/day for 16 weeks did not affect the MAP in SD rats, as shown in Fig. 26 below. The previous study from our lab with a 500 mg/kg/day for 4 weeks in Sprague-Dawley rats also showed no effect on the MAP and heart rate (Martin and Desai, 2020).



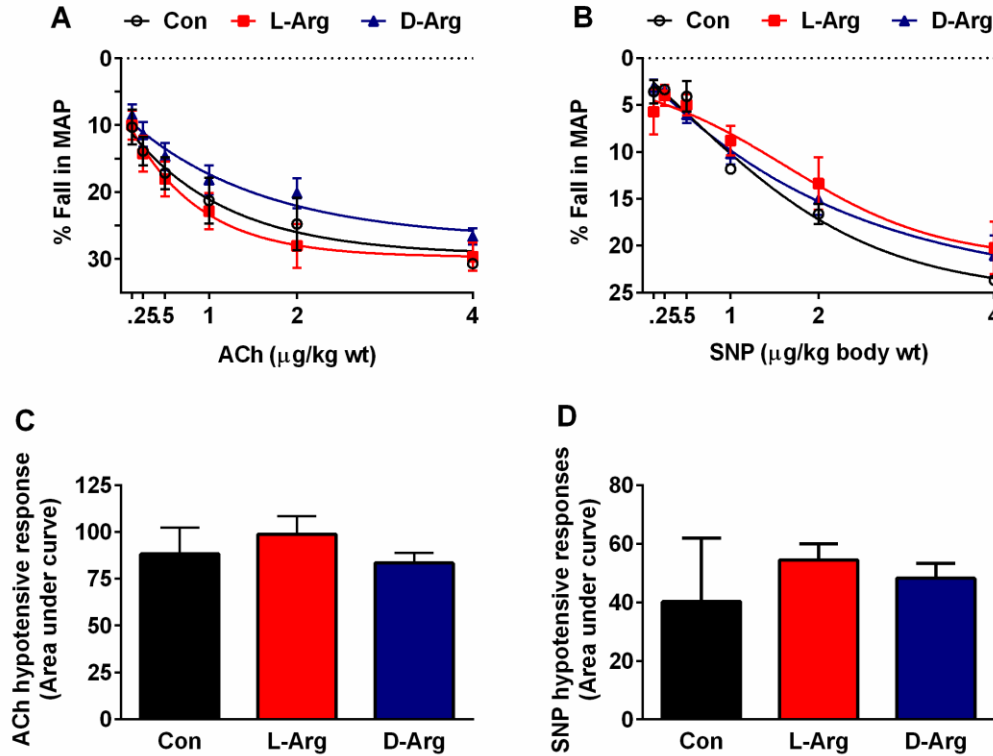
**Figure 26. Oral arginine did not affect the mean arterial pressure or heart rate in Sprague-Dawley rats.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. The mean arterial pressure (MAP) was measured in anesthetized rats with a carotid artery cannula as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups). (Unpublished data. With permission from my supervisor, Dr K Desai).

This result on the MAP is very significant because it questions the validity of taking arginine supplements for its presumed vasodilating effect by healthy people. However, oral L-Arg, but not

D-Arg, at a dose of 1000 mg/kg/day for 12 weeks given to male Zucker Diabetic Fatty rats, a model of type 2 diabetes with hypertension, significantly decreased the MAP in a study performed in our lab (unpublished results). This would suggest that arginine supplements might be beneficial in disease conditions, but not in healthy people. Some studies have shown beneficial effects of arginine supplements in people with hypertension (Lekakis et al., 2002), hypercholesterolemia (Clarkson et al., 1996) and T2DM (Lucotti et al., 2006). The exact mechanisms of how arginine supplements might benefit under disease conditions have not been elucidated.

Arginine supplements are popular in a major part due to their presumed NO-mediated vasodilatory effect. Our lab also examined the effects of L-Arg and D-Arg at 1000 mg/kg/day for 16 weeks given to SD rats, on acetylcholine-induced endothelium-dependent and sodium nitroprusside-induced endothelium-independent vasodilation. The results in Fig. 27 below show that neither L-Arg nor D-Arg affected ACh-induced or SNP-induced hypotensive responses. This again questions the validity of taking arginine supplements by healthy people for its NO-mediated vasodilation. Therefore, it is important to investigate the mechanism by which arginine supplements benefit people with cardiovascular disease such as hypertension and atherosclerosis, discussed above. The results of both MAP and endothelial function in healthy population are important to further investigate and understand, as some suggested that arginine supplements can reduce blood pressure in the healthy population as well, including those with only mild blood pressure elevation (Mayo Clinic, 2021). With this information, the healthy population may take these supplements for prevention of cardiovascular issues such as hypertension. In addition, something to consider is that it may be perhaps good if oral arginine does not affect the blood pressure in healthy population, as it could prevent complications associated with hypotension including dizziness and falls.

## Endothelium-dependent and -independent hypotensive responses



**Figure 27. Oral arginine did not affect the acetylcholine- and sodium-nitroprusside-induced hypotensive responses in Sprague-Dawley rats.** Nine-week-old male Sprague-Dawley rats were treated with L-arginine (L-Arg) or D-arginine (D-Arg) at a dose of 1000 mg/kg/day each in drinking water for 16 weeks. The control (Con) group received plain drinking water. The acetylcholine (ACh)-induced endothelium-dependent and sodium nitroprusside (SNP)-induced endothelium-independent hypotensive responses concentration-related responses were recorded in anesthetized rats with a carotid artery cannula as described in methods. The values are Mean  $\pm$  SEM. ( $n = 4$  for Con and  $n = 7$  each for L-Arg and D-Arg groups). (Unpublished data. With permission from my supervisor, Dr K Desai).

Arginase is the principal enzyme for arginine metabolism in the body, not only producing important products such as urea to detoxify ammonia, but is also competing with eNOS and downregulating it to cause various negative health effects associated with NO deficiency (Wu and Morris Jr, 1998). Both oral L- and D-Arg supplementation at a dose of 1000 mg/kg/day for 16 weeks in SD rats significantly increased arginase I expression in the liver in comparison to control (Fig. 12) and arginase II expression was decreased specifically with oral L-Arg supplementation in the ileum when compared with both the control and D-Arg groups (Fig. 12). In the kidney, neither L- nor D-Arg caused any significant changes in arginase II levels. This may be simply due to the fact that majority of the oral arginine gets metabolized prior to reaching the kidney, so that it is not able to cause any effects there. The observed decrease in arginase II expression in the ileum of SD rats treated with oral L-Arg was also seen with the lower dose study in our lab (Martin and Desai, 2020) and may have occurred from excessive amount of arginine acting upon arginase as a substrate, since arginase is the first and primary enzyme in the intestines for first-pass metabolism (Wu et al., 2007). The observed significant elevation of arginase I expression in the liver with the 1000 mg/kg/day dose of oral L-Arg seen in my study is not consistent with the effects of 500 mg/kg/day 4-week arginine treatment carried out in the previous study (Martin and Desai, 2020), where for this lower dose study, D-Arg had decreased the expression of arginase I in the liver compared to the control (Martin and Desai, 2020). The effect on liver arginase may be dose related, and could be explained by the decreased arginase II expression in the ileum, which would then allow more arginine to go to the liver and upregulate arginase I there. This finding is also consistent with the report that exogenous arginine induces arginase and offers one major reason why oral arginine supplements may not be beneficial (Dioguardi, 2011). Thereby, theoretically the more supplementation of arginine in the body, the more catabolic activity of arginase there would be. This may be problematic because, as mentioned earlier, upregulation of arginase causes negative effect on eNOS, suppressing the formation of NO, which results in various diseased conditions such as hypertension and endothelial dysfunction (Holowatz and Kenney, 2007; Caldwell et al., 2018). As to why an excess of a substrate would upregulate one enzyme and down regulate another one may be related to mechanism of regulation of a particular enzyme.

Additionally, L-Arg supplements also caused a significant decrease in arginase activity in the plasma in comparison to the control and D-Arg group (Fig.13A), whereas in the liver, ileum and the kidney (Fig. 13.B, C, D) it did not. This could be associated with our results on the effects of



L-Arg supplementation on arginase expression. Decreased arginase II expression in the ileum with oral arginine may lead to increased absorption of arginine in the systemic circulation. Subsequently, the increased bioavailable arginine in the blood may rapidly get distributed to various organs such as the liver through transporters, resulting in overall decreased levels of arginase activity in the plasma. In support, a study reported that chronic administration of L-Arg in Wistar rats increased plasma arginine concentration and decreased arginase activity (Moretto et al., 2017). In order to better understand the metabolic effects of oral arginine supplementation in the arginase metabolic pathway beyond the observation of the enzyme expression and activity, the levels of metabolites in this pathway, urea and hydroxyproline, were measured in the plasma and various organs (Fig. 14 and 15).

Urea levels remained unchanged with oral L-Arg or D-Arg supplementation in the plasma, liver and kidney in comparison to the control and with each other (Fig. 14A, C, D). However, urea levels were found to be significantly lower in the ileum with oral D-Arg than the levels resulting from L-Arg supplementation (Fig. 14B). This effect of D-Arg is unexpected and is difficult to explain, especially when D-Arg significantly increased urea levels in the skeletal muscle when compared with the L-Arg, but not the control group. In contrast in the skeletal muscle, urea levels were observed to be significantly lower for the SD rats treated with oral L-Arg than the levels found in D-Arg group of SD rats (Fig. 14E). In both cases, the ileum and the skeletal muscle, the significant differences produced by D-Arg were only in comparison to the L-Arg group and not the control group and so we can also say that L-Arg increased urea in the ileum and decreased it in the skeletal muscle in relation to D-Arg. It may be argued that the significance is reduced because the control group is not involved. Interestingly, D-Arg at 500 mg/kg/day for 4 weeks had significantly increased urea levels in the liver and the kidney, compared to the control group (Martin and Desai, 2020). Unfortunately, there is no clear explanation for this finding at the moment as pharmacodynamic studies on oral arginine and its effects on the skeletal muscle in association to urea production, are unable to be found.

L-proline is a product of L-ornithine catalyzed by OAT. To recall, L-ornithine is formed from L-Arg by arginase. When we investigated the effects of oral arginine on hydroxyproline levels, it was found that oral D-Arg, and not L-Arg supplements significantly decreased hydroxyproline levels in the ileum (Fig. 15B) and increased hydroxyproline levels in the brain (Fig. 15F), in

comparison to both the control and L-Arg groups of SD rats. In the plasma, liver, kidney, and the aorta however, the oral arginine treatment did not have any effect on hydroxyproline levels. These results reflecting the effects of specifically D-Arg supplements on hydroxyproline levels are again difficult to understand in connection to the resulting effects on arginase expression and activity with limited knowledge on the impact of D-Arg in the body. However, it is noteworthy that in the ileum, both urea and hydroxyproline levels were significantly decreased by D-Arg (Fig. 14B and 15B). This may possibly indicate that D-Arg plays a role in downregulating the arginase pathway in the ileum to lead to significant reduction in the metabolite levels, even though arginase II expression (Fig. 12) and activity (Fig. 13) were unchanged compared to control. In the long run, this may be problematic, as both urea and hydroxyproline play a significant role in the body, as described earlier.

Another major enzyme for arginine metabolism is NOS. Oral arginine supplements are primarily used for the effects of NO, produced from the NOS pathways. From the three isoforms of NOS, the focus of this present study was on eNOS, which is commonly known for its cardiovascular effects involved with vasodilation. As discussed earlier, the MAP as well as endothelium dependent vasodilation were not affected by arginine supplements, which is not surprising as several studies on arginine therapy have concluded that oral arginine is only effective in regulating blood pressure for people with cardiovascular diseases, and not in healthy individuals (Ast et al., 2011; Dong et al., 2011; Martin and Desai, 2020).

Oral D-Arg, but not L-Arg supplementation at 1000 mg/kg/day for 16 weeks significantly increased eNOS expression in the aorta and kidney of SD rats when compared with the control group (Fig. 16). This is interesting as no significant changes in eNOS expression occurred with L-Arg supplementation in those two organs, suggesting that there must be some selective effect of oral D-Arg on the eNOS pathway that is not known yet. Currently it is believed that D-Arg does not act as a substrate for eNOS (Palmer et al., 1988). A possible explanation for the upregulation of eNOS expression is similar as explained with CAT-1 expression in the ileum, that oral D-Arg may just have affinity and no metabolic effect on the eNOS, acting as a competitive inhibitor and preventing L-Arg from binding to the enzyme. With less enzyme availability for L-Arg to cause effects, enzyme expression might have been increased to make up for the reduced substrate-enzyme interaction. In comparison, in the previous study from our lab, L-Arg at 500 mg/kg/day

increased eNOS expression in the aorta compared to the control, and both, L-Arg and D-Arg at the same dose increased eNOS expression in the kidney compared to the control group (Martin and Desai, 2020). As to why a higher dose of L-Arg used for a longer duration in my study would not affect eNOS expression in the aorta and kidney is a matter of conjecture. Perhaps the body adjusts more with higher doses and longer duration of treatment, which hopefully will be proved with a dose of 500 mg/kg/day for 16 weeks in the third phase of this study in our lab.

In order to further investigate the effects on eNOS enzyme with oral arginine supplementation, a NOS activity assay was also performed in various organs such as the aorta, ileum, liver, kidney, lungs, brain and skeletal muscle. Despite the alterations in eNOS expression and its metabolite levels, the activity of NOS was not significantly affected by either isoform of oral arginine supplementation (Fig. 17). In the study with 500 mg/kg/day dose of L-Arg and D-Arg for 4 weeks, the NOS activity was not affected in most organs, except in the lungs where D-Arg reduced it significantly compared to the control and L-Arg groups (Martin and Desai, 2020).

Nitrate and nitrite are the main stable metabolic products of further NO metabolism. NO itself has a very short half-life of 5-6 sec and not easily amenable to measurement. We used an assay kit with nitrate reductase, which converted nitrate to nitrite which then was converted by the Griess reagent into a coloured product and measured. Fig. 18 shows nitrite levels, where L-Arg had significantly higher levels in the plasma, compared to D-Arg, but not control (Fig. 18A). Here again it is difficult to say that L-Arg nitrite levels were significantly higher because it was not in comparison to the control. In the previous study (Martin and Desai, 2020), L-Arg at 500 mg/kg/day for 4 weeks significantly increased nitrite levels in the plasma compared to control and D-Arg groups. The urinary nitrite levels were significantly lower in the L-Arg and D-Arg groups compared to control (Fig. 18B), which is difficult to explain, unless we use the theory that higher dose arginine supplements start utilizing the arginase pathway at the cost of NO production (Dioguardi, 2011). Thus, the reduced nitrite levels in the urine with oral L-Arg supplementation may be due to arginase reducing arginine bioavailability of L-Arg for eNOS, as arginase competes with eNOS for arginine (Wu, 2009) and arginase I has been found to be significantly upregulated in the liver in this study (Fig. 12). Another less likely explanation, which requires more proof, is that with oral D-Arg suppressing L-Arg interaction with eNOS, there will theoretically be less production of NO and excretion of nitrites from the body. In the ileum, oral D-Arg significantly

reduced nitrite levels in comparison to just the L-Arg group (Fig. 18C), which again raises the question whether D-Arg is metabolically inert as believed. With hardly any research on the effects of D-Arg in the metabolic pathways, and on the effects of chronic oral D-Arg supplementation in general at this very specific dose used in my study, a reliable interpretation on these results is not feasible at the moment.

ADMA is formed in the body during protein turnover and posttranslational modification where arginine is methylated. ADMA was first shown to be an endogenous inhibitor of eNOS, and its levels were increased in renal failure (Vallance et al., 1992). An ADMA assay was done in plasma and multiple organs of SD rats to investigate whether this played a role in the resulting levels of expression, activity and metabolites in the NOS pathway (Fig. 25). In result, ADMA levels in the plasma, liver, ileum, lungs and brain were not significantly affected by either of L-Arg or D-Arg administration. A 500 mg/kg/day dose of L-Arg and D-Arg for 4 weeks significantly increased ADMA levels in the kidney compared to the control and D-Arg groups (Martin and Desai, 2020). Studies looking at ADMA levels after arginine supplements are hard to find, but are very necessary since ADMA levels are associated with several disease conditions (Böger et al., 2000; Dayal and Lentz, 2005; Stühlinger and Stanger, 2005; Krzyzanowska et al., 2006) and it is important to know whether oral arginine supplements affect their levels. Bode-Boger *et al.* (Bode-Böger et al., 2003) reported that L-Arg supplementation at 8 g twice a day orally for 14 days given to people over 70 years of age improved endothelial function and while it did not alter ADMA levels it improved the L-Arg/ADMA ratio.

The AGAT pathway is essential for creatine biosynthesis in the body for regulation of energy balance (Wu and Morris Jr, 1998). Both oral L- and D-Arg supplements at 1000 mg/kg/day for 16 weeks significantly decreased AGAT expression in the liver, compared to the control group (Fig. 19), whereas in the kidney, oral L-Arg and not D-Arg led to a significant increase in AGAT expression, compared to the control and D-Arg groups (Fig. 19). As there were no assay kits to quantify the levels of the direct metabolite, creatine, creatinine levels instead were measured in plasma, urine, and various organ samples of SD rats. Creatinine is a degradation product of creatine. Creatinine levels were significantly decreased in the urine with both oral L-Arg and D-Arg supplementation but increased in the ileum with oral D-Arg, and not L-Arg supplementation (Fig. 20). Creatinine levels in the plasma, liver and kidney were not affected by arginine supplements

(Fig. 20). The reduction in creatinine levels in the urine as a result of oral arginine treatment may be due to the significant decrease in AGAT expression in the liver caused by both, L-Arg and D-Arg (Fig. 19). Excessive downregulation of AGAT in the liver could affect creatinine formation by attenuating creatine formation, and thereby, less creatinine would be excreted and detected in the urine. The effects of D-Arg treatment on creatinine levels again are surprising, and difficult to understand with such limited evidence of D-Arg as a metabolically active substrate in the body. In the previous study from our lab (Martin and Desai, 2020), L-Arg at 500 mg/kg/day for 4 weeks had significantly decreased creatinine levels in the skeletal muscle compared to the control group, whereas D-Arg at the same dose had increased it in the liver compared to the control group.

In the ADC pathway known for producing polyamines, ADC converts arginine into agmatine, subsequently followed by the conversion of agmatine into putrescine by agmatinase. This is an important metabolic pathway, as polyamines serve important roles in the body, such as cell growth and supporting embryo development (Wang et al., 2014a). However, ornithine, which is a metabolite of the arginase pathway is a major precursor for polyamines and the enzyme that converts it into polyamines, ODC, is the rate-limiting enzyme for *de novo* polyamine synthesis (Wang et al., 2014a). Therefore, ADC is referred to as the alternative enzyme for polyamine synthesis next to the primary enzyme, arginase. Both oral L- and D-Arg supplementation at 1000 mg/kg/day for 16 weeks in SD rats significantly increased ADC expression in the liver, compared to the control group, and significantly decreased ADC expression in the ileum compared to the control group (Fig. 21). This increase in ADC expression in the liver with oral L-Arg administration may have been due to extensive arginine metabolism with upregulated arginase I as first pass metabolism (Fig. 12), limiting arginine availability for ADC causing an adaptive increase in expression. In addition, the decreased expression of arginase II in the ileum with L-Arg supplementation (Fig. 12) may have played a role in increasing ADC expression in the liver to make up for the high levels of oral arginine reaching the liver, which has not been metabolized in the ileum. The 500 mg/kg/day oral L-Arg for 4 weeks had also significantly increased ADC expression in the liver (Martin and Desai, 2020). Oral L-Arg-induced decreased ADC expression in the ileum (Fig. 21) in my study is difficult to interpret when considering a decrease in arginase II expression in ileum as well with L-Arg supplementation. Theoretically, decreased arginase II in the ileum should lead to increased ADC expression to compensate for the oral arginine to be used

for polyamine synthesis. This suggests the complex interaction and physiological effects of oral arginine on its metabolic pathways.

The levels of agmatinase expression and total polyamines were measured in addition to determining ADC expression in various organs. Agmatine, the major metabolite of the ADC pathway is degraded by agmatinase to ultimately form various types of polyamines (Wang et al., 2014a). Interestingly, only the oral D-Arg, and not L-Arg, supplements caused significant effects in agmatinase expression in comparison to control or L-Arg groups (Fig. 22). In the kidney and ileum, D-Arg increased agmatinase expression compared to the control and/or L-Arg groups (Fig. 22), whereas in the brain it decreased agmatinase expression compared to the control group (Fig. 22). One possible mechanism for increased expression may be due to adaptive response, with supplemental D-Arg blocking L-Arg from interacting with ADC to form agmatine or D-Arg blocking agmatine from being catalyzed by agmatinase causing its upregulation. As D-Arg supposedly does not get used up in the body of mammals, the reasoning behind these results remains in question for now. We were not able to measure agmatine levels by HPLC because the protocol described in some literature reports (Zhao et al., 2002; Regunathan et al., 2009) could not be reproduced.

Oral L-Arg supplements significantly increased the total polyamine levels in the plasma, in comparison to both the control and D-Arg groups of SD rats (Fig. 23A). This can be explained by increased arginase I expression in the liver by both L-Arg and D-Arg (Fig. 12), which also contributes to polyamines formation through L-ornithine/OAD/putrescine pathway (Fig. 4). In comparison, D-Arg at 500 mg/kg/day for 4 weeks had significantly increased total polyamines in the plasma compared to the control, and L-Arg at the same dose had increased it in the liver compared to the control and D-Arg groups in SD rats (Martin and Desai, 2020).

The activity assays for the last two enzymes, ADC and AGAT, are currently not available during our search, and therefore were not able to be completed. The results from these assays would have been useful in filling the gap of knowledge in the mismatch of results observed with enzyme expression and metabolite levels of those two pathways and understanding the relationship between the two factors. For instance, the expression levels for an enzyme can be significantly elevated in a particular organ, but activity levels may be downregulated or upregulated by other factors. Understanding the activities of these enzymes would be helpful in viewing the overall

picture of what really happens at the molecular level in these pathways with oral arginine supplementation at 1000 mg/kg/day for 16 weeks.

#### 4.1 CONCLUSIONS

The main conclusion that can be drawn from my study is that oral arginine supplements with both the L and D isomers significantly affect the expression of its metabolizing enzymes and the levels of their metabolites in the plasma or some organs, though not in a uniform or consistent pattern (Table 1). This proves our hypothesis partly in that L-Arg will alter the expression/activity of its metabolizing enzymes and levels of their metabolites but disproves our hypothesis that D-Arg will not affect the metabolic pathways for arginine. This was also seen with a lower dose of 500 mg/kg/day of L-Arg as well as D-Arg administered for a shorter duration of 4 weeks in SD rats in the previous study from this lab (Martin and Desai, 2020). These results mandate that caution should be taken in the use of oral arginine supplements, especially by healthy people. They may not be as safe as advertised in media and as believed by the general public. The effects with both doses, 500 mg as well 1000 mg were not consistent which could be a result of multiple metabolic pathways for L-Arg, which probably interact in a more dynamic fashion with each other than shown by limited research in this area. It may be because of the rapid and dynamic metabolism that both doses showed changes in the plasma or organ levels of arginine. As summarized in Table 1, L-Arg significantly increased the expression of arginase I and ADC in the liver and AGAT in the kidney, and it decreased the expression of arginase II in the ileum and AGAT in the liver. In terms of their metabolites, oral L-Arg increased urea and hydroxyproline levels in the ileum, and nitrite and total polyamines levels in the plasma. L-Arg decreased urea nitrite and creatinine levels in the urine (Table 1). On the other hand, the supposedly inert D-Arg increased CAT-1 expression in the ileum, arginase I in the liver, eNOS expression in the aorta and the kidney, ADC expression in the liver and agmatinase expression in the kidney and the ileum. D-Arg decreased ADC expression in the ileum and agmatinase expression in the brain (Table 1). In terms of their metabolites, oral D-Arg increased hydroxyproline levels in the brain and creatinine levels in the ileum (Table 1). D-Arg decreased hydroxyproline levels in the ileum, and nitrite and creatinine levels in the urine (Table 1). Each enzyme has its own multifaceted regulation at the molecular level, which makes interpretation of the effects of oral L-Arg and D-Arg difficult on different enzymes and their metabolites, especially they also vary from organ to organ.

Hopefully, these results will inspire more studies on oral arginine supplements and their impact on physiology and safety/adverse effects, especially when it appears that the use of these supplements is heavily promoted and is increasing.

#### **4.2 SIGNIFICANCE OF STUDY**

L-Arg serves an important role in producing various important molecules in the body. One of the products, NO from eNOS, is essential for maintaining good blood flow and alleviating vessel stress, decreasing the risk of endothelial dysfunction. As endothelial dysfunction has a reciprocal causal relationship with diabetes, L-Arg may decrease the risk of developing or severity of the vascular complications of diabetes. On top of the vasodilatory role related to NO production, L-Arg has an additional direct function of scavenging methylglyoxal, a highly reactive glucose metabolite which induces type II diabetes in Sprague-Dawley rats (Dhar et al., 2011). This arginine study contributed significant data and knowledge about the overall impact of oral arginine on the level and expression of the important enzymes and their metabolic products, in the arginine metabolic pathways. In comparison to the previous study employing a 4-week administration of 500 mg/kg/day arginine (Martin & Desai, 2020), my study used a higher dose (1000 mg/kg/day) for a longer duration (16 weeks) to see if the changes noted in the earlier study are magnified and if further metabolic changes occur. While several changes observed in the metabolic pathways with a 500 mg/kg/day dose were not replicated with a 1000 mg/kg/day dose, there were some other changes, which reflect the dynamic and rather unpredictable regulation of arginine metabolism. At the same time, my study highlights the fact it is difficult to predict an optimum dose and duration oral arginine supplements, which does not adversely and significantly affect its metabolism in the body. Clearly, more studies are indicated employing different doses and treatment durations to form concrete use guidelines for oral arginine supplements. One thing for sure is that arginine supplements in the two doses used in our lab significantly affect several enzymes and metabolite levels and caution is required in the use of oral supplements, which seems to be lacking at the moment.

#### **4.3 LIMITATIONS OF STUDY**

The animal model used in my study, Sprague-Dawley rats, can be considered to be one of the limitations of this arginine study, as there are some physiological differences between a rat model



and a human model. Direct application of results from animal model study to real-life human use is deemed to be unreliable and invalid. However, the rat model is known for its relative similarity to human model in genes and pharmacokinetics. Thereby, this animal model study sets a baseline of pharmacological research in pre-clinical testing stage.

Another limitation is sample size chosen for each group of rats. In the previous lower dose arginine study (500 mg/kg/day for 4 weeks), there were 8 rats in each of three groups, whereas in the current study (1000 mg/kg/day for 16 weeks), 7 rats were used for each of the L-Arg and D-Arg groups and only 4 rats were used for the control group, with a view to reduce the number of rats used. In hindsight, this was an error because the duration of the current study was 16 weeks whereas it was only 4 weeks for the 500 mg/kg/day study (Martin & Desai, 2020). So results from the control group in the Martin & Desai study (2020) cannot be extrapolated to the control group in the current study. If there were 7 rats in each group including the control, the error bars could have been smaller and more significant values might have been detected, where it was borderline.

Additionally, in contrast to the lower dose oral arginine (500 mg/kg/day for 4 weeks) study (Martin & Desai, 2020), which used  $\beta$ -actin for loading control, I used total protein as a loading control for the current study. If the western blots were repeated with  $\beta$ -actin for the current study, the results perhaps could have been different and similar to the lower dose study. The supervisor's lab intends to repeat the western blots with  $\beta$ -actin as loading control.

Male rats were used for this study, which is a limitation because sex hormones might affect the results, and to avoid a confounding factor in interpreting results. Separate studies on female rats are planned as a part of this project in our lab.

The method of oral arginine administration remains to be another limitation to this study. The ideal method for administration is *via* oral gavage, instead of adding it in drinking water, for more precise delivery of the set dose of 1000 mg/kg/day, as well as for consistent and rapid absorption of arginine in the rats. In addition, human arginine supplements are mainly taken in pill form, which is a more concentrated dose, as seen with oral gavage administration. However, daily oral gavage for 16 weeks was not practical since it would add stress as a confounding factor in the analysis of results. It also increases the risk of pulmonary aspiration of the gavage solution and mortality. We took precautions to calculate the exact dose that each rat received based on its body weight and daily water intake throughout the study.

As well, the activity assays for the other two enzymes AGAT and ADC are currently not made available for research use, and therefore were not able to be completed. The results from these assays would have been useful for interpreting the results of enzyme expressions and metabolite levels of those two pathways and understanding the relationship between the two factors.

#### **4.4 FUTURE WORK**

One of the primary purposes of my study was to determine the physiological impact of oral arginine supplements. While my study contributed a significant amount of data and knowledge covering the 4 main metabolic pathways of arginine, it also highlighted the fact that the physiological impact of arginine supplements may not be related to the dose and duration of treatment within certain limits, when considering the previous study from this lab (Martin & Desai, 2020). This conclusion needs to be confirmed with a couple of more such comprehensive studies. In accordance, two more studies have been planned, one employing a dose of 500 mg/kg/day but with a greater duration of 16 weeks of treatment. The other one will employ 1000 mg/kg/day for a shorter duration of 4 weeks. Unfortunately, the Animal Ethics committee did not approve a dose of 2000 mg/kg/day citing a possible risk of pancreatitis. Another future goal of the arginine project in our lab is to develop oral arginine supplements as safe and effective methylglyoxal scavengers (Dhar et al., 2012), to attenuate the risk of formation of advanced glycation end products (AGEs) and development of vascular complications of diabetes, since methylglyoxal levels are elevated in diabetic patients and it is a major precursor of AGEs formation (Desai and Wu, 2007).

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